For my parents Mathilde and Wolfgang

For my sister Barbara

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Mitogenic signaling by $G_{q/11}$-coupled receptors

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ABBREVIATIONS

ADAM	a disintegrin and metalloprotease
AP-1	activator protein 1
Apaf-1	apoptotic protease activating factor-1
AR	adrenergic receptor
ASK	apoptosis signal-regulated kinase
ATF	activating transcription factor
Bcl2	B-cell follicular lymphoma
BMK	big MAPK
BSA	bovine serum albumin
CalDAG-GEF	calcium- and diacylglycerol-regulated guanine-nucleotide exchange factor
CaM	calmodulin
CaMK	Ca^{2+}/calmodulin-dependent kinase
cAMP	cyclic AMP
Cdc42	cell division cycle 42
Crk	Cas-related kinase
COS-7	*Cercopithecus aethiops* (monkey, African green)
Csk	C-terminal Src kinase
DAG	diacylglycerol
ddH_2O	bidestilled H_2O
DEPC	diethylpyrocarbonate
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EF	helixE- and helixF-containing Ca^{2+} -binding protein
EGFR	epidermal growth factor receptor
EGTA	ethylene glycol bis(2-aminoethylether)- N, N, N', N' -tetraacetic acid
ERK	extracellular signal-regulated kinase
FADD	Fas-associated protein with death domain
FAK	focal adhesion kinase
FasL	Fas (APO1/CD95) ligand
FSH	follicle stimulating hormone
GAP	GTPase-activating protein
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein

GPCR	G protein-coupled receptor;
Graf	GTPase regulator associated with focal adhesion kinase
Grb2	growth factor receptor-bound protein 2
GRP	gastrin-releasing peptide
GDP, GTP	guanosine-5` biphosphate, -triphosphate
GST	glutathion-S-transferase
HB-EGF	heparin-binding epidermal growth factor
HEK	human embryonic kidney
IGFR	insulin-like growth factor receptor
IP_3	inositol-1, 4, 5-trisphosphate
IPTG	isopropyl-1-thio-ß-D-galactoside
IQ motif	ilimaquinone motif
IRS	insulin receptor substrate
JNK/SAPK	c-jun N-terminal kinase/stress-activated kinase
LH	luteinizing hormone
LPA	lysophosphatidic acid
M1R, M2R	muscarinic acetylcholine receptor
MAPK	mitogen-activated protein kinase
MAPKAPK	MAPK-activated protein kinase
MEF	myocyte enhancer factor
MEK	MAPK/ERK kinase
MEKK	MEK kinase
MLK	mixed lineage kinase
MMP	matrix metalloproteinase
Mnk	MAPK-interacting kinase
Msk	mitogen- and stress-activated protein kinase
MT-MMP	membrane type-MMP
NF-κB	nuclear factor-κB
Nir	Pyk2 N-terminal domain-interacting receptor
$p70^{S6K}$	p70 S6 kinase
p^{130}Cas	p130 Crk-associated substrate
PAK	p21-activated kinase
PBS	phosphate-buffered saline
Pap	Pyk2 C-terminus associated protein
PC12	pheochromocytoma cells
PCR	polymerase chain reaction

PDGFR	platelet-derived growth factor receptor
PI3K	phosphatidylinositol 3- kinase
PIP_2	phosphatidylinositol 4, 5-bisphosphate
PLC	phospholipase C
PKC	protein kinase C
PKD	protein kinase D
PKN	protein kinase N
PMSF	phenylmethylsulphonyl fluoride
POD	peroxidase
Pyk2	proline-rich tyrosine kinase
PSGAP	PH and SH3 domain containing GAP
Rac	regulator of actin cytoskeleton
Rap	Ras p21 protein
RasGRF	Ras guanine nucleotide-releasing factor
RasGRP	Ras guanine nucleotide-releasing protein
Rb	retinoblastoma protein
RBD	Ras-binding domain
RGS	regulator of G protein signaling
ROK	Rho-associated coiled-coil-forming protein kinase
Rsk	p90 ribosomal S6 kinase
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription-polymerase chain reaction
SCLC	small cell lung cancer
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SH2 and SH3	Src homology domain
Shc	SH2 domain-containing α2-collagen-related
Sos	son-of-Sevenless
Src	Retrovirus-associated DNA sequences
Sprk	Src homology 3 domain-containing proline-rich kinase/ MLK3
TACE	tumor necrosis factor α–converting enzyme
TAK	TGFβ-activated kinase
TAO	thousand-and-one amino acid protein kinase
TBST	Tris base-buffered saline + Tween 20

TC21	R-Ras2
TCF	ternary complex factor
TEMED	N, N, N´, N´, -tetramethylethylenediamine
TGFα	transforming growth factor alpha
TIMP	tissue inhibitor of matrix metalloproteinase
TMPS	triple membrane passing signal mechanism
TNF	tumor necrosis factor
TRADD	TNF receptor-associated protein with death domain
TRAIL	TNF-related apoptosis inducing ligand
TRP	transient receptor potential
VGCC	voltage-gated calcium channel
V_m	membrane potential
VSMC	vascular smooth muscle cells
wt	wild type

1. INTRODUCTION

No cell lives in isolation. In all multicellular organisms, survival depends on an elaborate intercellular communication network that coordinates growth, differentiation and apoptosis of cells in diverse tissues and organs. Intercellular communication is accomplished by extracellular signaling molecules interacting with the cell by cell surface receptors. Binding of extracellular signaling molecules to cell surface receptors triggers intracellular pathways that ultimately modulate cellular development, differentiation, growth or metabolism. The multitude of cell surface receptors fall into four main classes: Ligand-gated ion channels, receptors linked to cytosolic tyrosine kinases, receptor tyrosine kinases (RTKs) harboring intrinsic catalytic activity and G protein-coupled receptors (GPCRs) (Gether 1998, Ji 1998, Strader 1994).

1.1. Signaling pathways emanating from G protein-coupled receptors (GPCRs)

GPCRs constitute a family of receptors that mediate most of the cell-cell communication in humans and are encoded by the largest gene family in most animal genomes (Marinissen 2001, Neves 2002). This large receptor family includes light-activated receptors (rhodopsins) in the eye and odorant receptors in the mammalian nose, numerous receptors for various hormones, neurotransmitters as well as paracrine and autocrine factors (Gudermann 2000, Mombaerts 1999). All GPCRs contain seven transmembrane-spanning regions with their N-terminal segment on the exoplasmatic and their C-terminal segment on the cytosolic face of the plasma membrane. Although these receptors are activated by different ligands and may mediate different cellular responses, they all utilize similar signaling pathways.

GPCRs signal by activating heterotrimeric signal transducing guanine nucleotide-binding proteins (G proteins). Ligand binding to GPCRs activates a G protein, which then activates an effector enzyme to generate an intracellular second messenger.

Trimeric G proteins are composed of three subunits, called α, β and γ. The α chain ($G\alpha$) binds and hydrolyzes GTP whereas the β and γ chains form a tight complex ($G_{\beta\gamma}$) tethering the α subunit to the plasma membrane. When stimulated by binding to a ligand-occupied receptor, $G\alpha$ exchanges its GDP for GTP causing its dissociation from $G_{\beta\gamma}$. Both the GTP-bound α subunit as well as the $\beta\gamma$ complex are capable of

transmitting messages to downstream targets. The GTPase cycle is completed when GTP is hydrolyzed to GDP by intrinsic GTPase activity of Gα (Neer 1995). The activating process is terminated when Gα-GDP reassociates with the βγ complex. GTPase activity of Gα is enhanced on the one hand by its target proteins, such as phospholipase Cβ (PLCβ), or on the other hand by regulator of G protein signaling (RGS) proteins. RGS proteins act as GTPase-activating proteins (GAPs). The activated receptor serves as a guanine nucleotide exchange factor (GEF) for the G protein by promoting GDP dissociation (Ishii 2003).

GPCR signaling pathways have a wide spectrum of functions including regulation of cell proliferation and differentiation, promotion of cell survival and modulation of cellular metabolism (Gudermann 2000). Four distinct subfamilies of heterotrimeric G proteins are recognized todate: $G\alpha_{q/11}$, $G\alpha_s$, $G\alpha_i$, $G\alpha_{12/13}$ (Neves 2002, Fig. 1). Receptor stimulation of $G\alpha_{q/11}$ proteins results in activation of PLCβ thereby generating two second messengers, diacylglycerol (DAG) and inositol- 1, 4, 5-trisphosphate (IP_3), which activate protein kinase C (PKC) and elevate intracellular calcium concentrations ($[Ca^{2+}]_i$), respectively (Clapham 1995, Divecha 1995). The latter signaling molecules are central players coupling $G_{q/11}$ proteins to the extracellular signal-regulated kinase (ERK) cascade (Grosse 2000b, Marais 1998, Wittau 2000). $G\alpha_s$ proteins stimulate adenylyl cyclase resulting in increased concentrations of cyclic AMP (cAMP) (Houslay 1997, Hurley 1999). Activation of G_i proteins regulates cellular effects through both α and βγ subunits. $G\alpha_i$ couples to inhibition of adenylyl clyclase whereas $G_{\beta\gamma}$, e. g. is involved in activation of G protein-coupled inwardly rectifying potassium channels, adenylyl cyclase, PLC isoforms or phosphatidylinositol 3-kinase γ (PI3Kγ) (Camps 1992, Logothetis 1987, Stephens 1994, Tang 1991). G_{12} and G_{13} proteins activate the monomeric GTPase Rho via GEFs. G_{12} activity leads to the induction of stress fibers and modulation of Na^+/H^+ exchanger. Activity of G_{13} results in actin cytoskeletal changes, modulation of Na^+/H^+ exchanger as well as activation of protein kinase D (PKD) and PI3K isoforms (DerMardirossian 2001).

The termination of receptor function is achieved in a process called receptor desensitization. It is usually controlled by receptor phosphorylation mediated by second-messenger kinases, such as PKA or PKC, or by a distinct family of GPCR kinases (GRKs). “Heterologous” desensitization is realized by second messenger kinases and results in phosphorylation and desensitization of receptors which are not occupied by agonists (Zamah 2002). “Homologous” receptor desensitization is mediated by the GRK/β-arrestin (β-arr) system. Receptor phosphorylation by GRKs

leads to binding of β-arrestin proteins to the intracellular face of the receptor. β-Arrestin binding results in G protein-uncoupling of GPCRs by sterically hindering receptor/G protein interactions and mediates targeting of the GPCR for endocytosis in clathrin-coated pits. Several GPCRs internalize as a stable complex with β-arrestin and the stability of this complex appears to regulate whether the receptors are dephosphorylated in early endosomes and recycled back to the cell surface as fully functional receptors, retained in early endosomes or targeted for degradation in lysosomes (Seachrist 2003).

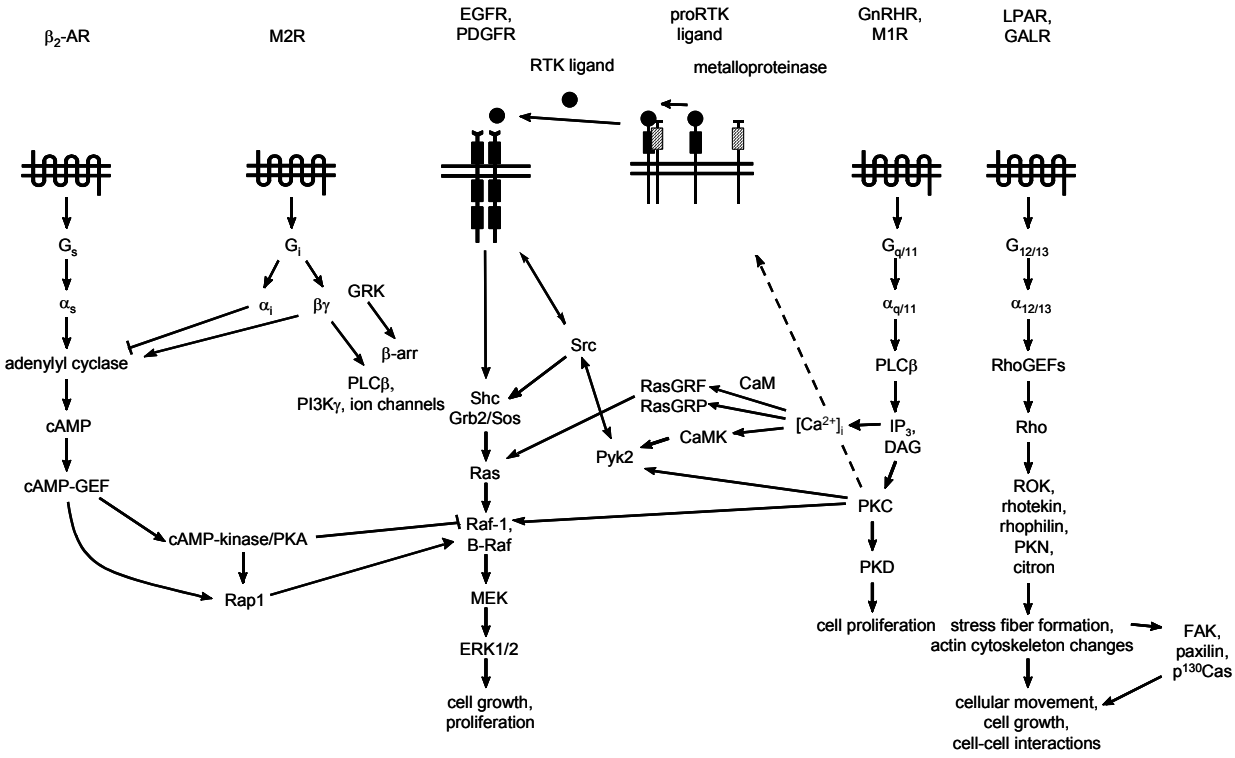


Figure 1. Mitogenic signal transduction emanating from G protein-coupled receptors.

After agonist binding, GPCRs couple to their cognate G proteins. The mitogenic input is then transmitted via a plethora of different signaling cascades. The dashed line represents a putative relationship. β2-AR, β2-adrenergic receptor; CaM, calmodulin; CaMK, Ca^{2+}/ calmodulin-dependent kinase; cAMP-GEF, cAMP-activated guanine nucleotide exchange factor; Crk, Cas-related kinase; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; GALR, galanin receptor; GnRHR, gonadotropin-releasing hormone receptor; Grb2, growth factor receptor-bound protein 2; LPAR, lysophosphatidic acid receptor; M1R, M2R, muscarinic acetylcholine receptor; MEK, mitogen-activated protein kinase/ERK kinase; p^{130}Cas, p130 Crk-associated substrate; PDGFR, platelet-derived growth factor receptor; PKA, protein kinase A; PKN, protein kinase N; Pyk2, proline-rich tyrosine kinase 2; Rap1, Ras p21 protein 1; RasGRF, Ras guanine nucleotide-releasing factor; RasGRP, Ras guanine nucleotide-releasing protein; ROK, Rho-associated coiled-coil-forming protein kinase; RTK, receptor tyrosine kinase; Shc, SH2 domain-containing α2-collagen-related; Sos, Son-of-sevenless, Src, Retrovirus-associated DNA sequences.

1.2. Mitogenic signaling by $G_{q/11}$-coupled receptors

GPCRs transduce mitogenic signals by engaging the ERK cascade. p42 ERK (ERK1) and p44 ERK (ERK2) belong to the superfamily of mitogen-activated protein kinases (MAPKs) (Pearson 2001, Fig. 2). MAPKs are a family of serine/threonine kinases, which become activated in response to mitogens, growth factors, cellular stress and neurotoxic factors (Chen 2001). So far, four highly conserved MAPK subfamilies are known: ERKs, c-Jun N-terminal kinase/stress-activated kinase (JNK/SAPK), p38 mitogen-activated protein kinase (p38MAPK) and big MAPK (BMK1/ERK5) (Chen 2001). GPCRs activate MAPK cascades by a variety of mechanisms (Gudermann 2000, Marinissen 2001, Pearson 2001). An intensive cross-talk between different classes of receptors, e. g. GPCRs and receptor tyrosine kinases (RTKs), such as platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR) or c-kit, contributes to an even wider range of cellular responses (Daub 1996, Herrlich 1998, Imokawa 2000). MAPKs are activated via a highly conserved kinase module consisting of three kinases (MEKK/MEK/MAPK, Fig. 2) which sucessively phosphorylate and activate the downstream component (Pearson 2001). In addition to agonists acting on GPCRs, ERK1 and ERK2 are predominantly activated by growth factors, such as epidermal growth factor (EGF) or platelet derived growth factor (PDGF). Activation of ERK1 and ERK2 by ligand-bound EGFR has been the best studied MAPK signaling cascade so far (Gudermann 2000). The EGFR family of receptor tyrosine kinases is ubiquitously expressed and consists of four members: EGFR/ErB1, HER2/ErB2, HER3/ErB3 and HER4/ErB4 (Ullrich 1984). Being activated by their six structurally related agonists- EGF, tumor growth factor α (TGFα), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, betacellulin and epiregulin- the receptors promote pathways entailing proliferation and transformation. Activated EGFRs homo- or heterodimerize and subsequently autophosphorylation of cytoplasmic tyrosine residues is initiated. These phosphorylated amino acids represent docking sites for a variety of different proteins (Prenzel 2001). Phosphorylated EGFR recruits adaptor proteins like Shc and Grb2 which become associated with the RasGEF Sos thus leading to GTP-loading of the small G protein Ras. The MEKK Raf is recruited to the plasma membrane by activated Ras and subsequently activates MEK eliciting p42 and p44 phosphorylation (Fig. 2). Activated ERKs target a diverse pool of proteins: they translocate to the nucleus activating transcription factors of the activator protein-1 (AP-1) family; phosphorylate a subset of protein kinases, e.g. Rsks, Mnks, Msks, MAPKAPKs,

which then activate downstream targets involved in transcriptional regulation or associate with cytoskeletal proteins (Pearson 2001, Reszka 1995, Shaulian 2002).

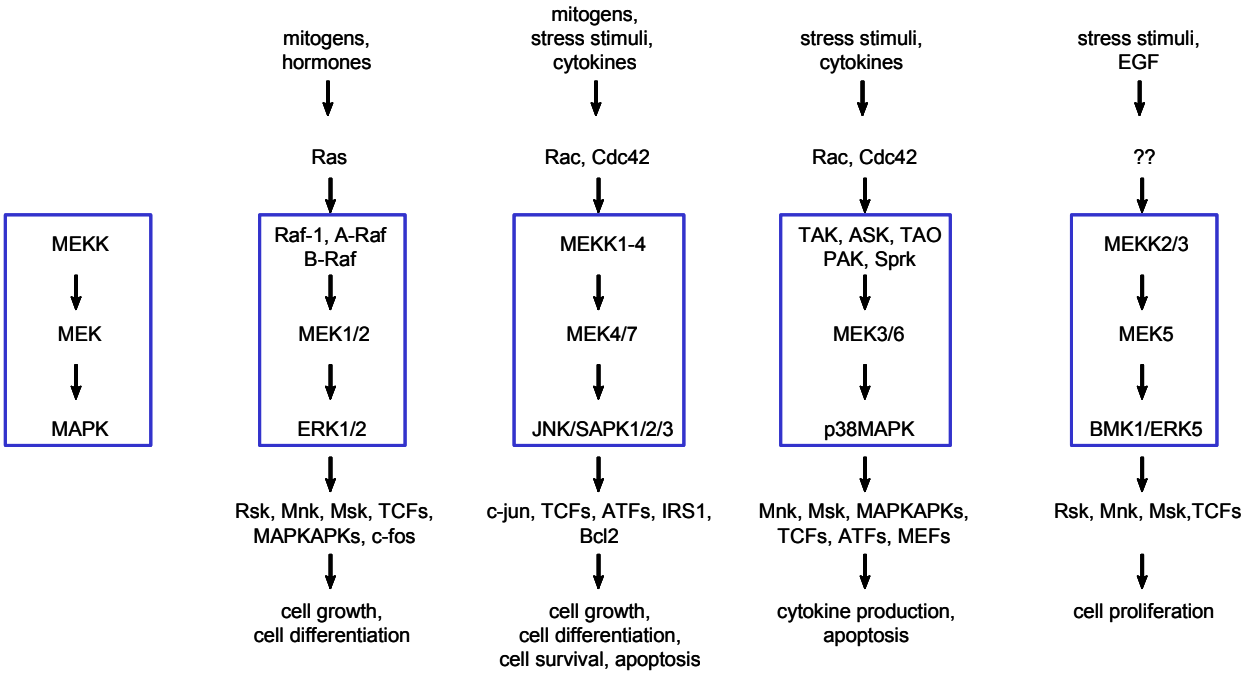


Figure 2. MAP kinase cascades.

MAPK cascades are composed of a a three-step module consisting of three conserved kinases which sucessively phosphorylate the downstream kinase. The protein located upstream of MEKK2/3 leading to BMK1/ERK5 activation has not been identified so far. ASK, apoptosis signal-regulated kinase; ATF, activating transcription factor; Bcl2, B-cell follicular lymphoma; BMK1, big MAPK; Cdc42, cell division cycle 42; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; IRS1, insulin receptor substrate; MAPK, mitogen-activated protein kinase; MAPKAPK, MAPK-activated protein kinase; MEK, MAPK/ERK kinase; MEF, myocyte enhancer factor; MEKK, MEK kinase; Mnk, MAPK-interacting kinase; Msk, mitogen- and stress-activated protein kinase; PAK, p21-activated kinase; Rac, regulator of actin cytoskeleton; Sprk, Src homology 3 domain-containing proline-rich kinase/MLK3; Rsk, p90 ribosomal S6 kinase; TAK, TGFβ-activated kinase; TAO, thousand-and-one amino acid protein kinase; TCF, ternary complex factor.

JNKs and p38MAPKs modulate cellular responses to a wide variety of extracellular stimuli including mitogens, inflammatory cytokines and stress stimuli (Chen 2001). In contrast to its ability to activate the ERK cascade, Ras only minimally affects JNK. However, overexpression of the constitutively activated mutants of the small G proteins, Rac and Cdc42, leads to robust stimulation of JNK activity (Coso 1995). The pathways leading to JNK activation mirror those seen for ERK with a variety of MEKs phosphorylating the various JNK isoforms (Pearson 2001, Fig. 2). As with ERKs, the end result of JNK activation is phosphorylation of certain transcription factors within their activation domains (Pearson 2001).

Increased BMK1 activity can be observed in response to growth factors and serum as well as stress stimuli (Chen 2001).

As mentioned above, agonist stimulation of $G_{q/11}$-coupled receptors leads to activation of PLCβ isoforms (Rebecchi 2000). All four mammalian PLCβ isoenzymes are activated by $G_{q/11}$ proteins to various degrees. Under the control of the receptor, PLCβ hydrolyzes the membrane lipid phosphatidylinositol 4, 5-bisphosphate (PIP_2) thereby generating two second messengers: diacylglycerol (DAG) and inositol- 1, 4, 5-trisphosphate (IP_3). DAG and IP_3 stimulate distinct downstream signaling pathways. DAG remains associated with the plasma membrane where it directly activates PKCs, a family of serine/threonine kinases contributing essentially to growth control and differentiation (Divecha 1995). Even though PKC isotypes are considered key enzymes linking $G_{q/11}$-coupled receptors to activation of the Raf/MEK/ERK cascade, reports concerning PKC downstream events are still rather inconsistent. ERK activation by agonists acting on α_{1B} adrenergic- and M1 muscarinic receptors proceeds by a pathway employing PKCα or PKCε, respectively, and Raf-1 - with PKCα and PKCε directly phosphorylating and activating Raf-1 (Hawes 1995, Kolch 1993). Nevertheless, it was demonstrated that activation of PKC leads to activation of Ras and formation of Ras/Raf-1 complexes. Since a dominant-negative Ras mutant did not block Raf-1 activation, a distinct mechanism of Ras activation from that engaged by RTKs may exist (Marais 1998). Furthermore, $G_{q/11}$-mediated ERK activation can be achieved exploiting PKC activation or EGFR transactivation in parallel pathways (Adomeit 1999). It has been reported that neither Ca^{2+}-release nor PKC activation are sufficient to induce ERK activation mediated by α_{1A} adrenergic receptor activation in adrenal pheochromocytoma (PC12) cells (Berts 1999).

On the contrary to DAG, IP_3 diffuses into the cytoplasm regulating increases of $[Ca^{2+}]_i$. Rises in $[Ca^{2+}]_i$ are realized by releasing Ca^{2+} from intracellular stores or by influx of extracellular Ca^{2+} through Ca^{2+}-channels (Berridge 2000). Intracellular Ca^{2+}-binding proteins, such as calmodulin (CaM), act as calcium sensors decoding the information according to distinct increases in $[Ca^{2+}]_i$ (Agell 2002).

In this context, Ca^{2+}-dependent proline-rich tyrosine kinase Pyk2 has been identified as an intermediate in Ca^{2+}-regulated ERK activation. After complex formation with Src tyrosine kinases, the Pyk2/Src complex recruits the Grb2/Sos complex to the plasma membrane causing GTP-loading of Ras and subsequent engagement of the ERK/MAPK cascade (Dikic 1996, Lev 1995, Yu 1996). A more direct connection between Ca^{2+} and ERK activation is provided by two families of Ca^{2+}-dependent RasGEFs, RasGRF and RasGRP, which can be differentiated by their cellular control

mechanisms (Cullen 2002, Table 1). Members of the RasGRF family are highly expressed in neurons. RasGRF proteins are IQ motif-containing GEFs with their activity being enhanced following binding of Ca^{2+}-bound CaM. RasGRF1 is activated indirectly through CaM binding to its IQ motif whereas Ca^{2+} might exert an additional stimulatory effect on a closely related exchange factor RasGRF2. A dominant-negative form of RasGRF1, GRFβ, is expressed in pancreatic β cells and seems to antagonize Ca^{2+}-dependent signaling of RasGRF1. RasGRP/CalDAG-GEF proteins contain a calcium binding EF hand and a DAG binding site and are indeed activated following direct binding of Ca^{2+} and DAG. CalDAG-GEF I prefers Rap1a over N-Ras and a splice variant of the same exchange factor, RasGRP2, is inhibited rather than stimulated by Ca^{2+} when functioning as a RasGEF. The production of DAG is the key factor of the spatio-temporal regulation of RasGRP3 and RaGRP4 whereas elevation of $[Ca^{2+}]_i$ does not affect the exchange activities of these GEFs (Arava 1999, Cullen 2002, Ebinu 1998, Fam 1997, Farnsworth 1995). The net effect of Ca^{2+} on Ras/ERK signaling is not only determined by GEF activities but also by Ca^{2+}-dependent deactivation utilizing GAPs. The two known directly Ca^{2+}-triggered RasGAPs are calcium-promoted Ras inactivator (CAPRI) and a related protein, Ras GTPase-activating-like (RASAL), preferentially expressed in endocrine tissue (Walker 2003). Furthermore, Ca^{2+}-induced ERK/MAPK activation can be achieved via EGFR transactivation.

Table 1. Ca^{2+}-regulated Ras exchange factors.

Name	Modulator	Effectors/effects	Tissue expression
RasGRF1	Ca^{2+} ↑, Ca^{2+}/CaM	H-Ras, R-Ras, Rac1 ERK/MAPK activation	neurons, pancreatic β cells
RasGRF2	Ca^{2+} ↑, Ca^{2+}/CaM	H-Ras, Rac1 ERK/MAPK activation	ubiquitous
mouse GRFβ		dominant-negative regulator of RasGRF1 in β cells	pancreatic β cells
RasGRP/CalDAG-GEF II	Ca^{2+} ↑, DAG↑↑	H-Ras, R-Ras	forebrain
CalDAG-GEF I	Ca^{2+} ↑, DAG ↑	Rap1a	forebrain
RasGRP2 (splice variant of CalDAG-GEF I)	Ca^{2+} ↑, DAG ↑	N-Ras, K-Ras	brain
RasGRP3/CalDAG-GEF III	DAG ↑	H-Ras, R-Ras, TC21, M-Ras, Rap1a, Rap2a ERK/MAPK activation	glial cells of the brain, kidney
RasGRP4	DAG ↑	H-Ras	blood leukocytes, myeloid cell lines

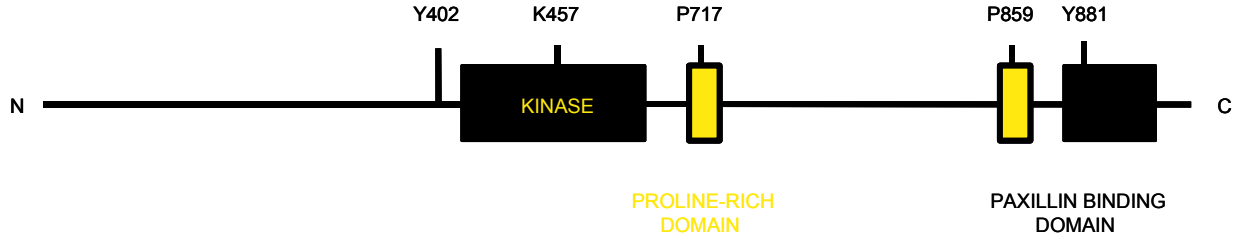
1.2.1. Ca^{2+}-mediated activation of Pyk2

Proline-rich tyrosine kinase Pyk2, also known as cell adhesion kinaseβ (CAKβ), related adhesion focal tyrosine kinase (RAFTK) or calcium-dependent protein-tyrosine kinase (CADTK), is a member of the focal adhesion kinase family of non-receptor kinases and was cloned from a human brain cDNA library. Pyk2 is predominantly expressed in the central nervous system and in hematopoietic lineage as well as in liver epithelial and vascular smooth muscle cells (Lev 1995, Yu 1996).

Pyk2 shares a high degree of sequence homology with focal adhesion kinase (FAK), especially in the kinase domain (~60%) and the C- and N-terminal domains (~40%) (Avraham 1995, Herzog 1996, Lev 1995, Yu 1996). The catalytic domain of Pyk2 is flanked by non-catalytic C- and N-termini with two proline-rich domains and a paxillin binding motif integrated in the C-terminus (Schlaepfer 1999, Fig. 3). Pyk2 lacks SH2 and SH3 domains but contains sequences capable of binding to SH2 and SH3 domains of other proteins as well as several tyrosine residues mediating specific protein-protein interactions (Dikic 1996, Qian 1997, Schlaepfer 1999). Tyrosine at position 402 has been identified as the major site for Pyk2 autophosphorylation and Src activation, with other tyrosine residues (579, 580 and 881) contributing to subsequent transphosphorylation and enhanced kinase activity (Schlaepfer 1999). Phosphorylation of tyrosine 881 creates a SH2 binding site for the adaptor protein Grb2 (Blaukat 1999). Pyk2 mutated at lysine 457 has been shown to be impaired in its ability to phosphorylate ion channels or glycogen synthase kinase 3β and is therefore appointed kinase-dead Pyk2 (Pyk2-PKM) (Hartigan 2001, Lev 1995). The two proline-rich motifs have been shown to be sites for SH3 domain-binding interactions, which are phosphorylation-independent. The SH3 domain of p^{130}Cas is constitutively bound to Pyk2 via the first proline-rich domain. RhoGAPs, Graf and PSGAP, preferentially bind to the second proline-rich domain of Pyk2 (Astier 1997, Ohba 1998, Ren 2001).

Pyk2 becomes activated in response to a variety of extracellular stimuli elevating $[Ca^{2+}]_i$ and/or activating PKC, such as agonists of GPCR, growth factors, stress stimuli as well as integrin-induced signaling pathways (Avraham 2000, Lev 1995, Tai 2002). Calcium signals responsible for Pyk2 activation can be generated by influx of extracellular calcium through calcium channels or through calcium release from intracellular stores (Lev 1995, Tokiwa 1996, Yu 1996). The proximal events coupling increases of $[Ca^{2+}]_i$ or PKC to Pyk2 activation are not yet understood. In this context, Ca^{2+}/CaM-dependent kinase II (CaMK II) has been implicated as an intermediate in Ca^{2+}-dependent activation of Pyk2 in VSMCs (Ginnan 2002). Determined by cellular

environment and activation mode, Pyk2 is engaged in ERK-, JNK- and p38MAPK activation (Lev 1995, Sorokin 2001, Tokiwa 1996, Yu 1996).



Pyk2	Site	Function
Y402	**Y**AEI	Src family tyrosine kinases SH2 binding
K457	AV**K**TCK	kinase region
Y579, Y580	ED**YY**KAS	Regulatory-/kinase activation loop
P717	PPPK**P**SRPK	p^{130}Cas SH3 binding site
P859	**P**PQKPPRLG	p^{130}Cas/Graf/PSGAP SH3 binding sites
Y881	**Y**LNV	Grb2 SH2 binding site
paxillin-binding domain	875-894, 985-999	paxillin binding

Figure 3. Schematic drawing of Pyk2.
Modified from Schlaepfer 1999.

Interestingly, it has been reported that several fibroblastic and epithelial cell lines, e.g. rat-1, Swiss 3T3 or HEK293 cells transfected with Pyk2, undergo apoptosis, whereas no detectable cell death could be observed in cells expressing significant high levels of endogenous Pyk2, like PC12 cells (Xiong 1997). In this regard Pyk2 has to be considered as a critical molecular switch in cell fate decisions.

1.2.2. GPCR-dependent Epidermal Growth Factor receptor transactivation

It had been noticed for quite some time that the EGFR became activated by stimuli other than its cognate ligands, namely agonists of GPCRs, cytokines, chemokines, other RTK agonists, cell adhesion elements as well as stress stimuli (Carpenter 1999). Time course experiments revealed that GPCR-induced EGFR activation occurs within short periods after agonist challenge (Daub 1996). In addition, rapid ERK activation by these agonists is achieved in an EGFR-sensitive mode and seems

to mimic those of RTKs (Daub 1996, Grosse 2000). Taken together, these data implicated a distinct activation mechanism from the one engaged by RTKs and was therefore referred to as “receptor transactivation”. Transactivation of the EGFR has been reported for G_q-, G_i, G_{13}- and recently for G_s-coupled receptors (Castagliuolo 2000, Gohla 1998, Grosse 2000b, Piiper 2002).

RTK transactivation was initially thought to be a ligand-independent pathway since several studies failed to detect released growth factors in culture media (Daub 1996, Eguchi 1998, Tsai 1997). However, in a seminal study, the involvement of metalloproteinase activity in the GPCR/EGFR cross-talk has been revealed (Prenzel 1999). Blocking of metalloproteinases using a broad-spectrum metalloproteinase inhibitor as well as blockage of the EGFR ectodomain with an antibody abolished EGFR activation induced by GPCR agonists. Ligand-dependent EGFR transactivation can be viewed as operating through a “triple membrane-passing signal mechanism” (TMPS, Fig. 1 and 4). Therein, agonists acting on GPCRs induce a metalloproteinase activity which cleaves an EGFR ligand precursor. The secreted mature ligand then activates the EGFR by binding to its extracellular domain (Gschwind 2001, Wetzker 2003).

EGFR agonists are commonly synthesized as membrane spanning pro-growth factors and are released through regulated metalloproteinase activity (Prenzel 2001). Enzymes which have been implicated in ectodomain shedding of growth factors mainly belong to two families: Matrix metalloproteinases (MMPs) and metalloprotease-disintegrin proteins/a disintegrin and metalloproteases (MDCs/ADAMs) (Massova 1998).

MMPs are a family of currently known 26 zinc- and calcium-dependent endopeptidases secreted by cells. MMPs are synthesized as zymogens with a signal sequence and a propeptide segment being removed during activation. After activation, MMPs are secreted into the extracellular medium except for the membrane type (MT)-MMPs which are tethered to the cell surface by a hydrophobic transmembrane domain (Werb 1997). MMPs regulate cell-matrix composition by degrading components of the extracellular matrix and are divided into 5 subgroups according to their substrate specificity and structure: collagenases, stromelysins, gelatinases, MT-MMPs and other MMPs. Physiologically, MMPs are implicated in a multitude of processes such as neurite growth, cell migration, bone elongation, wound healing, angiogenesis, ovulation, sperm maturation, menstruation, antigen processing and presentation and embryo implantation. Pathological processes involving MMPs include tumor growth and migration, fibrosis, arthritis, multiple sclerosis and infertility (Massova 1998). Endogenous regulators of MMP activities are

the tissue inhibitors of metalloproteinases (TIMPs). Four TIMPs are known so far harboring different inhibition efficiencies against MMPs. TIMPs have also been shown to inhibit ADAMs (Brew 2000). Synthetic MMP inhibitors are currently being developed and some have reached clinical trials as anti-cancer therapeutics (Wojtowicz-Praga 1996).

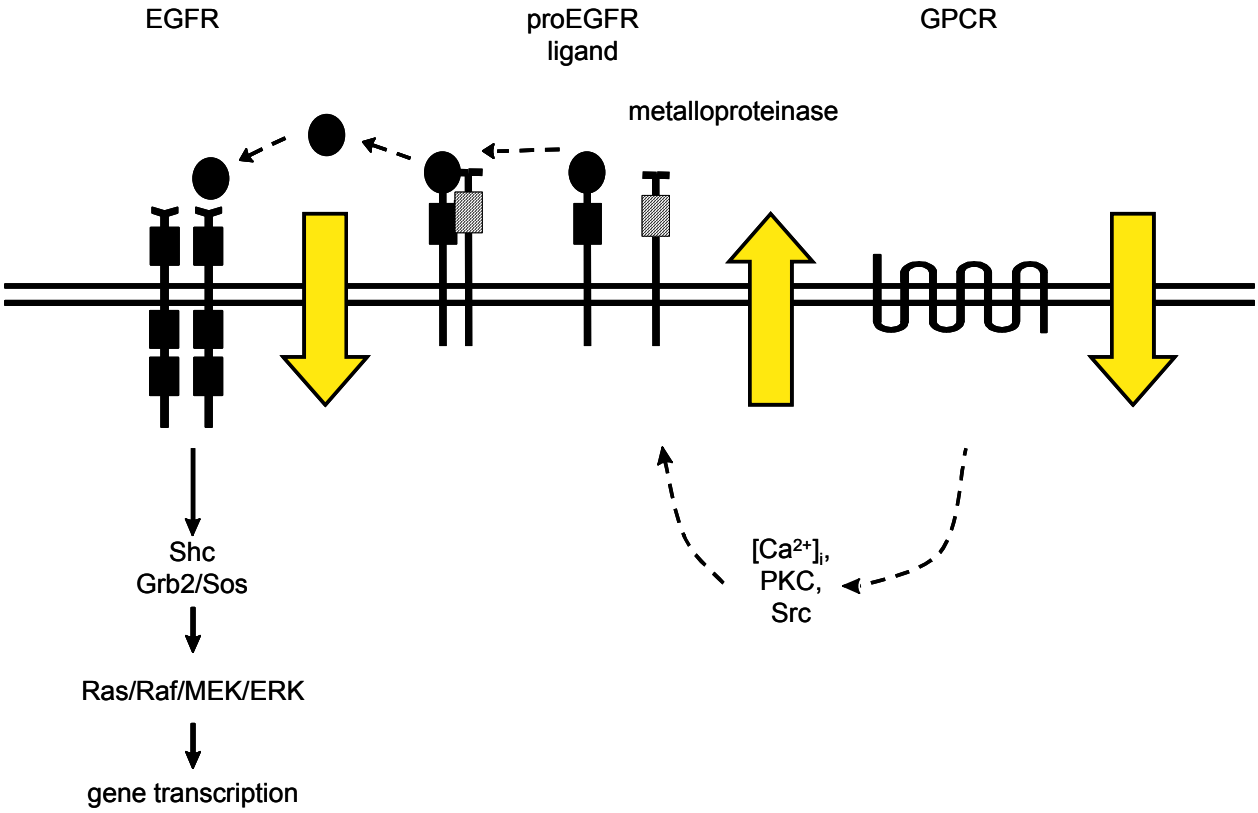


Figure 4. Triple membrane-passing signal mechanism of EGFR transactivation.

GPCR activation leads to metalloproteinase-mediated proteolytic cleavage of EGF-like growth factor precursors. Mature growth factors activate the EGFR. This model for ligand-dependent inter-receptor communication encloses three membrane passages as indicated by the yellow arrows.

The identity of the participating MMPs, their activation mechanism as well as their physiological relevance for GPCR-initiated signaling processes still remain elusive (Suzuki 1997). Recently, ADAM10, ADAM12 and ADAM17/tumor necrosis factor α–converting enzyme (TACE), three members of the ADAM family of proteases, have been claimed responsible for ectodomain shedding of EGF-like ligands resulting in EGFR transactivation. Platelet-activating factor receptor and ADAM10 mediate responses to *Staphylococcus aureus* leading to mucin synthesis and ADAM10 induces bombesin-triggered EGFR transactivation in COS-7 cells. In a cardiac cell model, ADAM12 is responsible for HB-EGF shedding-induced EGFR

activation by some vasoactive molecules (Asakura 2002, Gschwind 2003, Lemjabbar 2002, Yan 2002). In addition, ADAM9 (MDC9/meltrin-γ) has been identified as the protease responsible for the shedding of HB-EGF precursor in response to PKC activation (Asakura 2002, Gschwind 2003, Izumi 1998, Lemjabbar 2002, Yan 2002). However, GPCR-stimulated HB-EGF cleavage cannot be suppressed by dominant-negative forms of ADAM9, indicating that yet another protease mediates EGFR activation in response to GPCR signaling. ADAMs are transmembrane proteins characterized by a zinc-dependent metalloproteinase-, an adhesion-, a fusion- and an intracellular signaling domain (Blobel 1997). Cleavage of pro-growth factors, cytokines and cell surface receptors is mediated by ADAMs. ADAM functions have been linked to mammalian sperm-egg fusion, ectodomain shedding and diverse functions in Drosophila development (Blobel 1997). ADAM17/TACE represents the best known ADAM family member and was the first ADAM to have a known physiological substrate. The close resemblance of the severe phenotype of the ADAM17/TACE-deficient mice with that of EGFR knock out mice uncovered ADAM17/TACE as the protease responsible for ectodomain shedding of TGFα (Peschon 1998). TGFα has been shown to be an intermediate in the cross-talk between the prostaglandin E_2 receptor and the EGFR, suggesting that ADAM17/TACE might be the responsible metalloprotease (Pai 2002). Recently it was shown that ADAM17/TACE is responsible for cleavage of another EGFR-ligand, namely amphiregulin. Treatment of squamous cell carcinoma cells with LPA or carbachol induces ADAM17/TACE-dependent cleavage of proamphiregulin regulating proliferation and motility of this cancer model (Gschwind 2003).

Nevertheless, depending on the cellular context, EGFR transactivation can also occur by ligand-independent signal transduction pathways. Thrombin-activated protease-activated receptor-1 generates an intracellular signaling cascade that leads to ligand-independent EGFR receptor activation (Sabri 2002).

EGFR transactivation has been reported to be fully PKC-dependent, partially PKC-dependent or PKC-independent. Cross-talk between purinergic P2Y2 receptors and the EGFR in PC12 cells has been shown to be achieved via PKC with contributions of rises in $[Ca^{2+}]_i$. In the same cell system, Ca^{2+}-transients are sufficient to elicit EGFR activation after bradykinin challenge. Our laboratory has recently shown that the gonadotropin-releasing hormone receptor (GnRHR) transactivates the EGFR via a pathway solely depending on PKC in gonadotropic αT3-1 cells (Grosse 2000b, Soltoff 1998, Zwick 1997). The involvement of Src tyrosine kinases in the cross-communication between GPCRs and EGFR is unquestioned but not without

controversy as far as the localization of this non-receptor tyrosine kinase is concerned. In COS-7 cells, LPA treatment leads to EGFR-dependent ERK activation. Inhibition of Src kinases by the specific inhibitor PP1 diminished ERK activation whereas EGFR phosphorylation was unaffected (Daub 1997). In addition, inhibition of EGFR kinase activity by the selective tyrphostin AG1478 decreased angiotensin II-mediated Src phosphorylation in vascular smooth muscle cells (VSMCs) locating Src downstream of the EGFR whereas Src kinases acting upstream of the transactivated EGFR has been reported in the case of the β-adrenergic receptor in cardiac fibroblasts (Kim 2002, Touyz 2002).

EGFR transactivation-triggered p38MAPK activation has been reported for a variety of stimuli. Thrombin activates p38MAPK by an EGFR-dependent mechanism via PKC and increases in $[Ca^{2+}]_i$ in VSMCs (Kanda 2001). Angiotensin II-induced EGFR transactivation is required for p38MAPK stimulation in the same cell system (Eguchi 2001). So far there are no reports on GPCR-induced EGFR transactivation involved in JNK and BMK1/ERK5 signaling.

1.3. Neuropeptide-mediated ERK activation

Neuropeptides are a structurally and functionally diverse group of molecular messengers that function as neurotransmitters, paracrine/autocrine regulators or systemic hormones in normal as well as cancerous cells (Rozengurt 2002). In the central nervous system, neuropeptides are responsible for regulating food intake, body temperature and behavioral responses. Throughout the organism they regulate exocrine and endocrine secretion, smooth muscle contraction, pain transmission, fluid homeostasis, blood pressure and inflammation (Rozengurt 2002). It is now well recognized that neuropeptides represent the most important growth factors in a variety of human cancers, most notably small cell lung cancer (SCLC) (Rozengurt 1999).

Receptors for neuropeptides belong to the large superfamily of heptahelical GPCRs (Gudermann 1997). It has been delineated that neuropeptide receptors couple to an assortment of G proteins. For example, in our laboratory the galanin receptor was shown to couple to $G_{q/11}$, $G_{i/0}$ and $G_{12/13}$ proteins (Wittau 2000). Pathways initiated by neuropeptides resulting in mitogenic responses are primarily related to G_q proteins and are known to signal via the PLCβ pathway thereby activating PKC as well as increasing $[Ca^{2+}]_i$ (Sethi 1992, 1993). Even though PKC plays a pivotal role in neuropeptide signal transduction, events downstream of PKC are still not well

understood. PKC, activated through neuropeptides, has been implicated in cell cycle events via the Raf/MEK/ERK as well as PKD pathways (Rozengurt 1998, Zhukova 2001). In contrast, it was shown that neuropeptide-mediated Ca^{2+}-transients are sufficient to trigger ERK activation in PC12 cells (Zwick 1997). Concordantly, a rise in $[Ca^{2+}]_i$ was shown to be necessary and sufficient to elicit neuropeptide-induced ERK activation in small cell lung cancer cells besides PKC activation (Wittau 2000).

1.3.1. The Gonadotropin-releasing hormone receptor utilizes PKC to activate the ERK/MAPK cascade

Typically, heptahelical receptors couple to an assortment of different G proteins making it difficult to link resulting cell responses to a certain G protein-coupling propensity. We resorted to the gonadotropin-releasing hormone receptor (GnRHR) because of some of its noteworthy features. In transfected COS-7 and αT3-1 cells derived from the gonadotrope lineage, the GnRHR initiates multiple signaling pathways by exclusively coupling to $G_{q/11}$ proteins (Grosse 2000a). Furthermore, the GnRHR completely lacks a cytosolic C-terminus and therefore is not phosphorylated by GRK. Internalization of GnRHR occurs extremely slow not coinciding with agonist challenge, and expression of the GnRHR at the plasma membrane is regulated in a β-arrestin-independent fashion (Grosse 2000a, McArrdle 2002). As stated before, ERK activation following GnRH stimulation in gonadotropic cells is solely PKC-dependent. Therefore, the GnRHR in αT3-1 cells served as a modell to investigate neuropeptide-mediated ERK activation via PKC.

Gonadotropin-releasing hormone (GnRH) is a neuropeptide (pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-amide) which is produced in neurosecretory cells of the hypothalamus. After secretion into the hypophysal portal circulation, GnRH binds to its cognate high-affinity receptor on pituitary cells thereby initiating signaling cascades leading to regulation of GnRHR number, synthesis and secretion of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Pierce 1981). The gonadotropins act on ovaries and testes to stimulate steroidogenesis and gametogenesis. LH and FSH belong to the glycoprotein hormone family and display a heterodimer structure composed of a common α subunit and a hormone-specific β subunit with physiological specificity. Specific GnRH pulse frequencies are necessary for determination of LHβ or FSHβ gene activation (Kaiser 1997). Still, the mechanism as to how gonadotropes differentiate between GnRH pulse frequencies and subsequently differentially regulate the

expression of the LHβ and FSHβ genes remains yet to be determined. It was recently shown that GnRHR numbers on gonadotropic cells seem to play a critical role in this process (Bedecarrats 2003).

The structural diversity of GnRH variants in vertebrates has been apparent for quite some time with 14 distinct peptides known so far (Kang 2003, Millar 2001). Among these, GnRH II, originally cloned from chicken brain, is expressed universally in mammals including humans (Kang 2003, Miyamoto 1984). By using a double-labeled histochemistry approach, it was revealed that the two GnRH types are expressed by two distinct populations of hypothalamic cells in mammals (Latimer 2000). The high expression of GnRH II outside the brain corroborates the belief that GnRH II might display functions distinct from those of GnRH (Lumpkin 1987, Okada 2003, Padmanabhan 2001). Recently, the GnRH II-receptor (GnRH II-R) has been cloned from monkeys and characterized which, in congruence to the GnRHR, is G protein-coupled. The receptor is functional and specific for GnRH II in terms of inositol phosphate-, LH- and FSH production (Millar 2001, Neill 2001). However, it has not been possible so far to identify a full-length transcript of GnRH II-R in human tissues (Neill 2002).

The majority of peptides mediate stimulation of cell growth with an essential contribution of ERK activity. However, GnRH-mediated stimulation of ERK activity in the pituitary is necessary for the maintenance of the gonadotropic phenotype (Kaiser 1997). In gonadotropic αT3-1 cells, all four subfamilies of MAPKs are activated upon GnRH stimulation, and ERK activity is required for GnRH-induced expression of the common α subunit gene through a pathway critically involving PKC (Kaiser 1997, Naor 2000, Reiss 1997, Roberson 1995, Sundaresan 1996).

Our laboratory has recently shown that GnRHR/$G_{q/11}$-coupling leads to rapid stimulation of ERK activity in transfected COS-7 and gonadotropic αT3-1 cells (Grosse 2000b). This effect can be fully mimicked by short-term phorbol ester (TPA) treatment. The involvement of PKC was further substantiated by inhibition of PKC using a specific inhibitor or by down-regulation of PKC by long-term TPA treatment leading to abrogation of GnRH-stimulated ERK activity. Elevation of $[Ca^{2+}]_i$ did not have any influence on GnRH-induced ERK activation as demonstrated by the inability of calcium ionophores to induce or calcium chelators to block GnRH-triggered ERK activity. ERK activation was sensitive to PP1 treatment and expression of Csk revealing Src as an intermediate signal transducer. Most notably, the kinetics and maximal response of ERK activation critically depends on EGFR transactivation (Grosse 2000b).

As described above, GnRH is the key hypothalamic signal peptide governing reproductive function. In addition to its “classical” hypophysiotropic action, GnRH has been implicated to modulate the activities of some extrapituitary tissues, such as brain, placenta, ovarian cells, myometrium and lymphoid cells as well as tumors arising from these tissues (Chegini 1996, Ho 1995, Kang 2003, Merz 1991, Peng 1994). It has been suggested that GnRH functions as an autocrine/paracrine factor in these cells (Gruendker 2002, Kraus 2001). However, data concerning GnRHR expression, regulation and mode of action in pheripheral organs and tumor cells are controversial and not fully understood (Kang 2000, Nathwani 2000). Experiments using antisera against GnRH caused significant increases in cell proliferation of 4/6 ovarian cancer cells, suggesting GnRH as a negative regulator of proliferation (Emons 2003, Voelker 2002). Nonetheless, opposing data exist for another ovarian cancer cell line, ES-2 cells, where GnRH acts as an autocrine growth factor (Arencibia 2000). GnRH II also has been shown to have an anti-proliferative effect on ovarian cancer cells (Choi 2001). The reason as to why GnRHR exploits different signal transduction pathways depending on the cell type remains to be discovered. It has been suggested that GnRHR might couple to pertussis toxin-sensitive G_i proteins in extrapituitary tissue leading to activation of phosphotyrosine phosphatases (PTPs). PTP might counteract the growth stimulatory properties of agonist-bound EGFR resulting in reversion of mitogenic signal transduction and cell proliferation (Gruendker 2001). Furthermore, NFκB as well as JunD are activated by GnRH in ovarian cancer cells protecting cells from apoptosis or leading to cell cycle arrest in $G_{0/1}$ (Gruendker 2000, Gunthert 2002). One may speculate that GnRH enables the tumor cell to acquire repair mechanisms while proliferation as well as apoptosis are repressed.

1.3.2. Galanin mediates growth of small cell lung cancer cells via rises in $[Ca^{2+}]_i$ and ERK activation

Due to over one million cases of lung cancer diagnosed every year, lung cancer is the leading cause of cancer death in the world (Parkin 2001). Human lung cancers are classified into two major groups, small cell lung cancer (SCLC) and non-small cell lung carcinoma (N-SCLC) with the latter consisting of several types (Travis 1995). Adenocarcinoma is the most frequent of the N-SCLC subtypes and is the most common in women and never smokers. On the contrary, SCLC is a neuroendocrine tumor that is strongly smoking-associated. Approximately 25% of all lung neoplasms are histologically classified as SCLC which is characterized by rapid growth and a

high metastatic potential. Despite initial radio- and chemosensitivity, SCLC possesses a 5-year survival rate of less than 5% (Clark 1998, Laskin 2003). Because SCLC tumors are seldom surgically resected, fresh tumor tissue is difficult to obtain and continuous cell lines are usually utilized for laboratory studies. Key events in the pathogenesis of SCLC are discrete mutations in tumor suppressor genes and chromosomal alterations like loss of heterozygosity of the short arm of chromosome 3 (3p[14-25]) in 100% of the cases examined (Lindblad-Toh 2000). There is almost universal loss of the tumor suppressors p53 and Rb and frequent amplification of oncogenes such as *myc* and *bcl2* in SCLC (Wistuba 2001). In contrast to N-SCLC, transforming Ras mutations or overexpression of RTKs are usually not encountered in SCLC, and the expression of GTPase-deficient Ras mutants in SCLC cells induces cell differentiation and growth arrest (Mabry 1988, 1989, Rozengurt 1999). Furthermore, overexpression of constitutively active Raf-1 leads to apoptosis in SCLC cells (Ravi 1998). On the contrary, fundamental evidence showed that broad-spectrum antagonists acting on neuropeptide hormone receptors efficiently block the growth of SCLC cells (MacKinnon 1999, Maruno 1998, Seckl 1997, Sethi 1992). These findings lend credence to the notion that calcium-mobilizing neuropeptides, e.g. bombesin/gastrin-releasing peptide (GRP), bradykinin, neurotensin, vasopressin and galanin, acting through autocrine and paracrine loops, represent the main driving force for the growth and metastasis of this tumor entity (Rozengurt 1999, Gudermann 2000). Neuropeptides are stored in cytoplasmic neurosecretory granules which are fused with the plasma membrane in response to Ca^{2+} stimuli.

PLCβ is critically involved in neuropeptide-stimulated growth of SCLC cells since expression of the catalytically inactive enzyme results in inhibtion of ERK activity and cell growth (Beekman 1998). In addition, PKC became evident as a central molecule for SCLC cell growth and some effort has been made to identify critical downstream effectors of PKC (Paolucci 1999, Seufferlein 1996b). In this regard it was shown that one major role of PKC in SCLC cells is the activation of the ERK cascade, and downregulation of PKC isoforms was shown to inhibit both ERK activity and anchorage-independent growth (Seufferlein 1996a). There is evidence that neuropeptides like galanin or bombesin strongly activate PI3K and the downstream located ribosomal p70 S6 kinase ($p70^{S6K}$), which has been implicated in regulation of translation, and that this pathway is important to maintain the anchorage-independent phenotype of SCLC cells (Moore 1998). It was further suggested that Src tyrosine kinases are not involved in this pathway, although no direct proof could be provided (Moore 1998). Our laboratory has recently shown that a rise of $[Ca^{2+}]_i$ is necessary and sufficient to elicit galanin-induced ERK activation in SCLC cells (Wittau 2000).

Therefore we concluded that calcium mobilization, besides contributing to PKC activation, may regulate other targets to stimulate ERK activity in SCLC cells. Therefore, SCLC cells served to investigate the mechanism underlying neuropeptide-induced ERK activation via rises of $[Ca^{2+}]_i$.

Due to their neuroendocrine origin, SCLC cells express dihydropyridine-sensitive L-type as well as dihydropyridine-insensitive P/Q-type and N-type voltage-gated calcium channels (VGCCs) (Watkins 2003, Williams 1997). Approximately 2-3% of SCLC patients present with Lambert-Eaton myasthenic syndrome (LES) caused by autoantibodies against P/Q-type VGCCs (Elrington 1991, Sutton 2002). LES is characterized by a generalized deficit of neurotransmitter release. Neurotransmitter release from presynaptic neurones at the neuromuscular junction and postganglionic sympathetic and parasympathetic neurones is mediated via depolarization of P/Q-type VGCCs. Autoantibodies that interfere with neurotransmitter release by binding to presynaptic VGCCs have been found in sera of patients with LES and autoantibodies from patients affected by LES can precipitate these channels. These autoantibodies also block Ca^{2+} influx through VGCCs in SCLC cells and thereby inhibit tumor growth. In fact, SCLC patients with LES had a significantly longer median survival time from the diagnosis of SCLC when compared with SCLC-only patients (Maddison 1999).

Neuropeptide-dependent proliferation of SCLC cells critically relies on the fine-tuned activation of two parallel signaling pathways (Fig. 5): By way of $G_{q/11}$/PLCβ activation the Ras/Raf/MEK/ERK cascade is engaged in a PKC- and/or Ca^{2+}-dependent manner. Amongst other effects, $G_{12/13}$ proteins mediate activation of RhoGTPases which impinge on the dynamics of the actin cytoskeleton and may contribute to the recruitment of the JNK cascade. JNK, however, can also be activated via $G_{q/11}$-dependent RhoGTPases. Selective engagement of the JNK pathway, for instance by biased neuropeptide receptor ligands, is invariably associated with apoptotic cell death (Chan 2002). Mechanistically, JNK-induced cell death of SCLC cells still remains enigmatic.

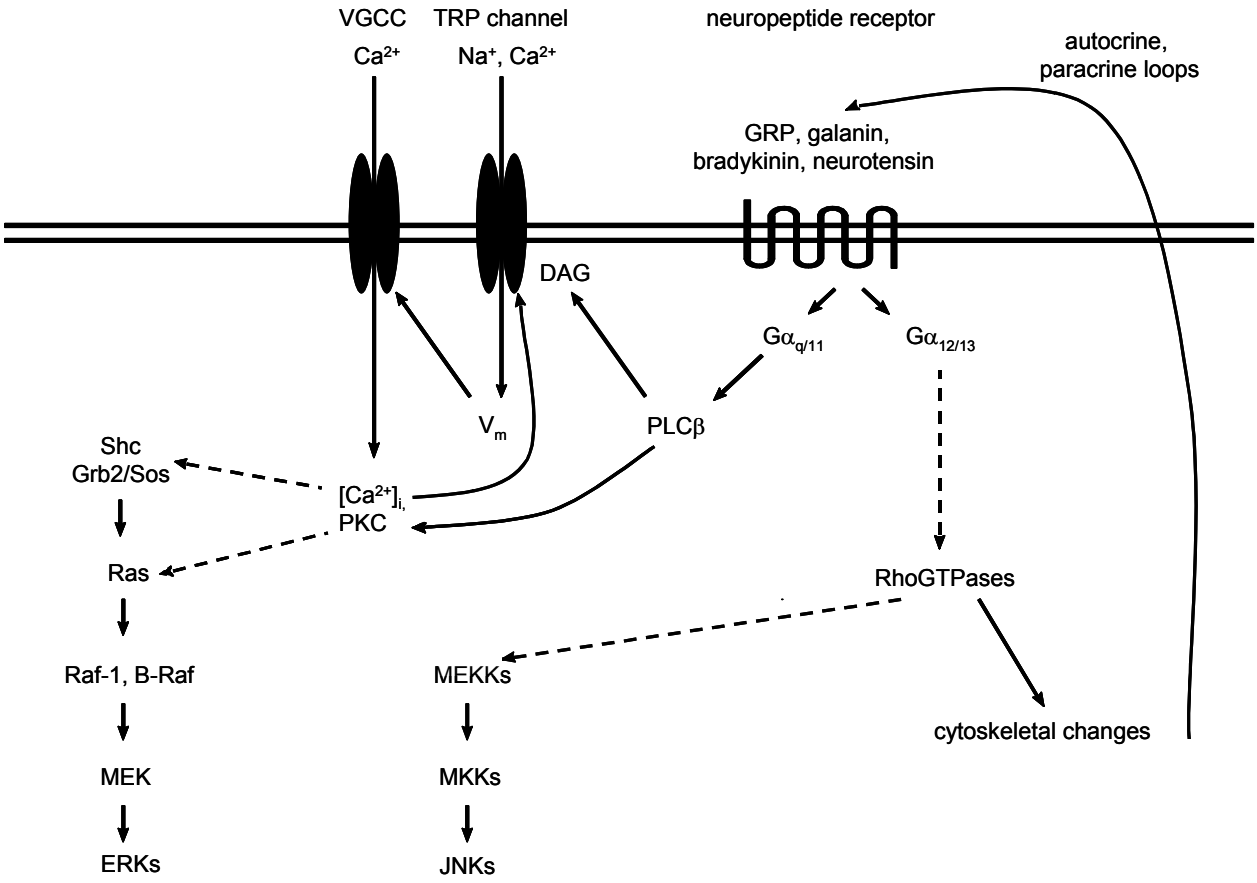


Figure 5. Current view of growth regulation of SCLC cells.

Apart from VGCCs, SCLC cells express ligand-gated cation channels, store- and receptor-operated as well as Ca^{2+}-activated cation channels. Members of the transient receptor potential (TRP) family of cation channels are likely candidates for receptor-operated cation entry. Cation influx is required to depolarize the cell thereby leading to VGCC-mediated Ca^{2+} infllux. Neuropeptides are stored in cytoplasmic neurosecretory granules which are fused with the plasma membrane in reponse to Ca^{2+} stimuli.

1.4. Apoptosis

Apoptosis is a normal physiological process of programmed cell suicide which is important both in maintenance of adult tissues and in embryonic development. Apoptosis is the complementary mechanism to cell proliferation ensuring homeostasis of all tissues. In addition, programmed cell death provides a defense mechanism by which damaged and potentially dangerous cells can be eliminated for the well-being of the organism as a whole. Apoptosis can be triggered in mature cells by external stimuli such as growth factor depletion, loss of contact with neighboring cells or adhesion, glucocorticoids, radiation exposure or chemotherapeutics (Raff 1998, Vaux 1999). Studies of cancer cells have shown that both uncontrolled cell

proliferation and failure to undergo programmed cell death can contribute to neoplasia and insensitivity to anti-cancer treatments (Reed 1999).

Programmed cell death is characterized morphologically by blebbing of the plasma membrane, volume contraction, condensation of the cell nucleus and cleavage of genomic DNA by endogenous nucleases into nucleosome-sized fragments. Such apoptotic cells and cell fragments are phagocytosed by macrophages and neighboring cells, efficiently removing dead cells from tissues. In contrast, cells that die as a result of acute injury swell and lyse, releasing their contents into the extracellular space and causing inflammation.

Three major apoptotic pathways have been elucidated todate all being orchestrated by caspases (Shivapurkar 2003). Caspases comprise a family of cysteine (C) proteases with high specificity for aspartatic residues (ASP) (Stennicke 2000). Caspases are expressed as inactive zymogens that can be activated by removal of the regulatory prodomain at the onset of apoptosis. Broadly, caspases can be divided into initiator (upstream) and effector (downstream) caspases (Shivapurkar 2003). Initiator caspases, such as caspases-8, -9 and –10 become activated by proapoptotic stimuli and subsequently activate effector caspases. “Executioner” caspases-3, -6 and –7 are responsible for cleaving apoptotic target proteins including inhibitors of DNase, laminin or cytoskeletal proteins (Earnshaw 1999).

One apoptotic pathway responds to death-inducing ligands such as TNF, FasL or TRAIL. After binding to their receptors, adaptor proteins FADD, TRADD and procaspase-8 are recruited to form a Death Inducing Signaling Complex (DISC). Recruitment of caspase-8 to the complex results in its proteolysis and subsequent activation of effector caspase-3 (Ashkenazi 1999). The closely related caspase-10 has been shown to additionally participate in DISC formation and both caspases seem to further play important roles in apoptosis initiation (Kischkel 2001).

The mitochondrial/cytochrome C pathway is largely mediated through Bcl2 family members. Release of cytochrome C from mitochondria results in formation of a complex called apoptosome containing adaptor protein Apaf-1 and procaspase-9 (Adrain 2001). Procaspase-9 subsequently becomes activated resulting in caspase-3 activation (Green 1998). Cytochrome C-mediated caspase-3 activation can be inhibited by inhibitor of apoptosis proteins (IAPs) which can be relieved by another mitochondrial protein, Smac/Diablo, by binding to the IAPs and releasing active caspases from their inhibitory influence (Du 2000, Verhagen 2000).

Finally, granzyme B directly cleaves and activates several caspases resulting in cell death (Lord 2003).

2. OBJECTIVES

Neuropeptides are responsible for a plethora of cellular functions in the central nervous system and in the periphery. They are also potent mediators for normal cell growth, differentiation and development. Furthermore, it is now well documented that neuropeptides play a crucial role in tumor growth via autocrine and paracrine growth loops. The most prominent example for a growth-stimulatory role of neuropeptides is represented by the growth regulation of small cell lung cancer cells.

Neuropeptides bind to GPCRs with a wide coupling-spectrum. Mitogenic signaling of neuropeptides, however, is achieved via the $G_{q/11}$/PLCβ/Ras/ERK pathway. In this signaling cascade activated PLCβ generates two second messengers, DAG and IP_3, which stimulate discrete downstream signaling pathways by activating PKC isoforms or regulating increases of $[Ca^{2+}]_i$, respectively.

The aim of this work was to analyze differences between PKC- or Ca^{2+}-mediated signaling cascades leading to ERK activation by neuropeptide stimulation.

The receptor for the neuropeptide gonadotropin-releasing hormone has been delineated as an exclusively $G_{q/11}$-coupling receptor in gonadotropic αT3-1 and transfected COS-7 cells. In these cells, $G_{q/11}$ activation leads to PKC-mediated EGFR transactivation resulting in a rapid stimulation of ERK activity. The project intended to investigate the cellular mechanism underlying PKC-induced EGFR transactivation in αT3-1 cells.

PLCβ-dependent elevation of $[Ca^{2+}]_i$ is the key event to initiate ERK activation in SCLC causing cellular proliferation of this tumor entity. By means of the galanin and bradykinin receptors in SCLC cells, the project aimed at delineating Ca^{2+}-dependent ERK activation in SCLC cells. Furthermore, Ca^{2+}-regulated target proteins in SCLC cells were to be identified.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Reagents

Name	Supplier
30% acrylamide/0.8% bisacrylamide	Roth
Bisbenzimide (Hoechst 33258)	Boehringer Mannheim
ECL sytem	Amersham Pharmacia Biotech
Expand™ High Fidelity	Boehringer Mannheim
jetPEI	Polytransfection
Metafectene	Biontex
poly-(L)-lysine	Fluka
QuikChange® multi site-directed mutagenesis kit	Stratagene
restriction endonucleases	New England Biolabs
Roti Block	Roth
SuperSript™ reverse transcriptase	Gibco BRL
TOPO TA cloning® kit	Invitrogen
Trizol	Gibco BRL
All other reagents were purchased from Sigma.	

3.1.2. Cell Culture Supply

Name	Supplier
Dulbecco´s modified Eagle´s medium (DMEM), high glucose	PAA
DMEM, low glucose	PAA
fetal calf serum (FCS)	PAA
gentamycin	PAA

Name	Supplier
Ham´s F12	PAA
horse serum (HS)	PAA
Phosphate-buffered saline (PBS)	PAA
RPMI 1640	PAA
streptomycin/penicillin	PAA
trypsin/EDTA	PAA

3.1.3. Antibodies

Name	Supplier
anti-c-fos, rabbit polyclonal	Santa Cruz Biotechnology
anti-c-jun, mouse monoclonal	Transduction Laboratories
anti-c-src (B12), mouse monoclonal	Santa Cruz Biotechnology
anti-c-src (Src2), rabbit polyclonal	Santa Cruz Biotechnology
anti-EGFR (sc-03), rabbit polyclonal	Santa Cruz Biotechnology
anti-ERK1/2 (K23), rabbit polyclonal	Santa Cruz Biotechnology
neutralizing anti-HB-EGF	R&D Systems
anti-MMP2 (SA103), rabbit polyclonal	Biomol
anti-MMP9 (SA106), rabbit polyclonal	Biomol
anti-mouse POD	Sigma
anti-MT1-MMP, rabbit polyclonal	Chemikon
anti-phosphoEGFR (Y845), rabbit polyclonal	Biosource International
anti-phosphoERK, rabbit polyclonal	New England Biolabs
anti-phosphoJNK, rabbit polyclonal	Promega
anti-phosphop38, rabbit polyclonal	New England Biolabs
anti-PI3K p85α (B-9), mouse monoclonal	Santa Cruz Biotechnology

Name	Supplier
anti-PI3K p110α (H-201), rabbit polyclonal	Santa Cruz Biotechnology
anti-PI3K p110β (N-20), rabbit polyclonal	Santa Cruz Biotechnology
anti-phosphoSrc (Y418), rabbit polyclonal	Biosource International
anti-phosphotyrosine (4G10), mouse monoclonal	Upstate Biotechnology
anti-Pyk2, mouse monoclonal	Transduction Laboratories
anti-rabbit POD	Sigma
anti-Ras (pan-ras), mouse monoclonal	Oncogene Research Products

3.1.4. Agonists and Drugs

Name	Supplier
AG1478	Calbiochem
BAPTA/AM	Molecular Probes
batimastat (BB-94)	Roche Diagnostics GmbH
bisindolylmaleimide II (GF109203X)	Calbiochem
galanin, porcine	Calbiochem
ionomycin	Calbiochem
LY294009	Calbiochem
PD98059	Calbiochem
phorbol-12-myristoyl-13-acetate (TPA)	Calbiochem
PP2	Calbiochem
Ro28-2653	Roche Diagnostics GmbH
Ro32-7315	Roche Diagnostics GmbH
All other reagents were purchased from Sigma.	

3.1.5. Oligonucleotides

Pyk2-1fwd	5′-GGCTGAGTGCTATGGGCTGAGGCTG-3′
Pyk2-1rev	5′-TCCCAAAGCAGATGCAGGAGAACTT-3′
Pyk2-2fwd	5′-AGGACATTGCCATGGAGCAAGAGA-3′
Pyk2-2rev	5′-CCCTGAGTGAGGAGTGCAAGAGGC-3′
rasGRF-fwd	5′-GCGGACAATGCCCACTGCTCT-3′
rasGRF-rev	5′-GTCCGCCACTGCCACCCATTTCT-3′
rasGRP-fwd	5′-GGACGCCAGCTTGACAGACAC-3′
rasGRP-rev	5′-CCCGGGCATAGGAAAGCTCATAGAT-3′
Pyk2-1818-F	5′-CCCGGTACATTGAGGACGAGGACTATTA-3′
Pyk2-3184-B	5′-GCAGAAGGCGGGGACGGACGGTA-3′
Pyk2-29-F	5′-GGGTGCCCGAGGAGTAGTCG-3′
Pyk2-2387-B	5′-ATTGAGTGACGTGTGGGGTGGAGAGGTGG-3′

3.1.6. Mutagenesis primer

Pyk2-DN-F	5′-CGTCTCTGGCGACCTGCAAGAAAGACTG-3′
Pyk2-DN-B	5′-CAGTCTTTCTTGCAGGTCTCTGC-3′
Pyk2mut-402fwd	5′-AGTCAGACATCTTCGCAGAGATTCC-3′
Pyk2mut-402rev	5′-GGAATCTCTGCGAAGATGTCTGACT-3′
Pyk2mut-717fwd	5′-CCCCCACCCAAGGCCAGCCGACCTAAG-3′
Pyk2mut-717rev	5′-CTTAGGTCGGCTGGCCTTGGGTGGGGG-3′
Pyk2mut-859fwd	5′-CCGCCACAGAAGGCCCCGAGGCTGGGC-3′
Pyk2mut-859rev	5′-GCCCAGCCTCGGGGCCTTCTGTGGCGG-3′

3.1.7. Hammerhead ribozymes

Rz-MMP2	5′ - CCACAGUGCUGAUGAGUCCGUUUAGGGACGAAACAUAGCG - 3′

Rz-MMP9	5´-ACGUCUGGCUGAUGAGUCCGUUAGGACGAAACACCACA – 3´
Rz-MMP12	5`-UGAUGGUGCUGAUGAGUCCGUUAGGACGAAACUGCUAG - 3´
Rz-MT1-MMP	5´-CUCAUUUUGCUGAUGAGUCCGUUAGGACGAAACAGUCCA - 3´
Rz-ADAM10	5´-CAAUGACACUGAUGAGUCCGUUAGGACGAAACCCAUGA - 3´
Rz-FGF-BP	5`-UUCCAAUACUGAUGAGUCCGUUAGGACGAAACUCUCU - 3´

3.2. Methods

3.2.1. Cell culture and transient transfection

3.2.1.1. SCLC cell lines

SCLC cell lines NCI-H69 and NCI-H510 (hereafter referred to as H69 and H510) were a kind gift of E. Rozengurt (School of Medicine, University of California, Los Angeles, CA). SCLC cell lines were grown in RPMI 1640 medium supplemented with 10% (v/v) FCS. The medium contained 50 units/ml penicillin, 50 μg/ml Streptomycin and 5 μg/ml L-glutamine. Cells were cultured in a humidified atmosphere of 5% CO_2/95% air at 37°C. Approximately 3 x 10^4 cells were used for experimental purposes. Prior to stimulation, cells were grown in HITESA medium for 3-5 days. Stimulation of cells was performed as described in the figure legends and reactions were stopped by transferring cells on ice.

Transient transfection of SCLC cells was carried out using Metafectene as a transfection reagent. Prior to transfection, SCLC cells were allowed to settle on poly-(L)-lysine-coated coverslips in 6-well plates. 0.8 µg DNA was added to 50 µl serum-free RPMI 1640 medium and mixed with 3 µl Metafectene prior diluted in 50 µl serum-free RPMI 1640 medium per well. To identify transfected cells, green fluorescent protein (0.2 µg peGFP-C1 in pcDNA3.1) was coexpressed as a marker. The mixture was incubated at room temperature for 15 min and subsequently added to the cells as indicated in the figure legends. Cells were incubated with the DNA/Metafectene mixture for 36 h and then subjected to fluorescence microscopy.

HITESA medium	
RPMI 1640 medium supplemented with	
10 nM hydrocortisone	10 nM estradiol
5 μg/ml insulin	30 nM selenium
10 μg/ml transferrin	0.25% BSA

3.2.1.2. Gene transfer into SCLC cells by a viral approach using retroviruses

Pyk2-wt, Pyk2-Y402F and Pyk2-PKM were cloned into the retroviral expression vector pLXSN (see 3.2.3.5.2.). Amphotrophic packaging cell line PA317 was transiently transfected with pLXSN, pLXSN-Pyk2-wt, pLXSN-Pyk2-Y402F or pLXSN-Pyk2-PKM using the calcium phosphate precipitation procedure (Hitt 1995). PA317 cells were exposed to the DNA precipitate until day 3 when the medium was aspirated and fresh medium was added. On day 4, the virus-containing medium was removed, centrifuged at 3000 x g for 5 min and virus titers of approximately 10^6/ml were utilized to infect H69 cells. Retrovirus-infected H69 cells were selected in G418 (100 μg/ml)-containing RPMI medium. Viability of H69 cells was controlled every day by trypan blue staining.

3.2.1.3. PC12W cells

PC12W cells were a kind gift of S. Bottari (Transduction Laboratory, Medical School, Université de Grenoble I, France). PC12W cells were cultured in DMEM, low glucose/Ham´s F12 (1:1, v/v) supplemented with 10% (v/v) HS, 5% (v/v) FCS, 5 μg/ml L-glutamine and 50 μg/ml gentamycin. Cells were cultured in a humidified atmosphere of 5% CO_2/95% air at 37°C. Transient transfection of PC12W cells was carried out using jetPEI as a transfection reagent. Prior to transfection, cells were seeded into 6-well plates and grown to 80% confluency. 0.8 μg DNA was added to 50 μl 150 mM NaCl pH 7.4 and mixed with 3 μl jetPEI prior diluted in 50 μl 150 mM NaCl pH 7.4 per well. To identify transfected cells, green fluorescent protein (0.2 μg peGFP-C1 in pcDNA3.1) was coexpressed as a marker. After vortexing, the mixture was incubated at room temperature for 15 min and subsequently added to the cells as indicated in the figure legends. Cells were incubated with the DNA/jetPEI mixture for 36 h and then subjected to fluorescence microscopy.

3.2.1.4. αT3-1 cells

αT3-1 cells were a kind gift of P. L. Mellon (School of Medicine, University of California, San Diego, CA). αT3-1 cells were grown in DMEM, high glucose supplemented with 5% (v/v) FCS, 5% (v/v) HS, 50 units/ml penicillin, 50 µg/ml streptomycin and 5 µg/ml L-glutamine. They were cultured in a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells were seeded into 6-well plates and grown to 80% confluency. Prior to stimulation, cells were serum starved for 6 h. Experiments were performed in serum-free medium containing various substances as indicated in the figure legends. Basal values were determined in serum-depleted medium.

Transient transfection of αT3-1 cells was performed using jetPEI as a transfection reagent. The indicated amount of ribozymal RNA was added to 50 µl 150 mM NaCl pH 7.4 and mixed with 3 µl jetPEI prior diluted in 50 µl 150 mM NaCl pH 7.4 per well. After vortexing, the mixture was incubated at room temperature for 15 min and subsequently added to the cells as indicated in the figure legends. Cells were incubated with the RNA/jetPEI mixture as specified and then cultured on serum-free medium for another 6 h before stimulation.

3.2.1.5. LβT2 cells

LβT2 cells were a kind gift of P. L. Mellon (School of Medicine, University of California, San Diego, CA). LβT2 cells were maintained in DMEM, high glucose supplemented with 10% (v/v) FCS, 50 units/ml penicillin, 50 µg/ml streptomycin and 5 µg/ml L-glutamine. They were cultured in a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells were seeded into 6-well plates and grown to 80% confluency. Prior to stimulation, cells were serum starved for 18 h. Experiments were performed in serum-free medium containing various substances as indicated in the figure legends. Basal values were determined in serum-depleted medium.

3.2.1.6. PC-3 cells

PC-3 cells were purchased from the American Tissue Culture Collection. PC-3 cells were grown in RPMI 1640 medium supplemented with 50 mM Hepes, 10% (v/v) FCS, 50 units/ml penicillin, 50 µg/ml streptomycin and 5 µg/ml L-glutamine. They were cultured in a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells were seeded into 6-well plates and grown to 80% confluency. Prior to stimulation, cells

were serum starved for 36 h. Experiments were performed in serum-free medium containing various substances as indicated in the figure legends. Basal values were determined in serum-depleted medium.

3.2.2. Biochemical Methods

3.2.2.1. Standard procedures

Detection of protein quantities	Bradford 1976
SDS-PAGE	Laemmli 1970
Western Blot	Towbin 1979

3.2.2.2. Immunoprecipitation

Cell stimulation was performed as indicated in the particular figure legends. For tyrosine phosphorylation of Pyk2 and coimmunoprecipitation experiments with Src kinases in SCLC cells, reactions were terminated by transferring cells on ice. After centrifugation at 300 x g, cells were lysed in 500 µl modified RIPA buffer for 15 min on ice. Lysates were cleared by centrifugation, supernatants diluted with 300 µl PBS and subjected to immunoprecipitation overnight at 4°C using the appropriate antibodies. Pyk2 was immunoprecipitated using monoclonal anti-Pyk2 antibody. For detection of coprecipitated Src kinases, monoclonal anti-Src antibodies were used. Subsequently, 100 µl of prewashed protein A-sepharose beads (12.5%, w/v) were added and the lysates incubated at 4°C at constant rotation for 2 h. Immune complexes were precipitated by centrifugation and washed three times with ice cold modified RIPA buffer. Precipitates were resuspended in 2x SDS-PAGE sample buffer, boiled for 3 min and resolved on 9% SDS-PAGE.

For immunoprecipitation of the EGFR, αT3-1 cells were lysed in 500 µl ice cold lysis buffer for 15 min. Lysates were precleared by centrifugation and supernatants diluted with 500 µl lysis buffer containing 0.1% Triton X-100. The EGFR was immunoprecipitated using a polyclonal anti-EGFR (sc-03) at 4°C overnight. Subsequently, 100 µl of prewashed protein A-sepharose beads (12.5%, w/v) were added and the lysates incubated at 4°C at constant rotation for 2 h. Immune complexes were precipitated by centrifugation and washed three times with ice cold lysis buffer. Immuncomplexes were resuspended in 2x SDS-PAGE sample buffer, boiled for 3 min and resolved on 9% SDS-PAGE.

Modified RIPA buffer

50 mM Tris-HCl, pH 7.5	1 mM Na_3VO_4
150 mM NaCl	20 mM NaF
0.3% deoxycholic acid	25 µM $ZnCl_2$
0.1% Nonidet P-40	1 mM PMSF
1.5 mM $MgCl_2$	10 µg/ml aprotinin
1 mM EDTA	10 µg/ml leupeptin
0.2 mM EGTA	

Lysis buffer

50 mM Tris-HCl, pH 7.5	500 µM sodium orthovanadate
100 mM NaCl	1% Triton X-100
5 mM EDTA	1 mM PMSF
1 mM dithiothreitol	1 µM leupeptin
40 mM $Na_4P_2O_7$	0.1 µM aprotinin

3.2.2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting procedures

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate complex protein mixtures by their molecular size. The standard Laemmli method (Laemmli 1970) is applied for discontinuous gel electrophoresis under denaturing conditions in the presence of SDS. Western blotting (immunoblotting) is applied to identify specific proteins (antigens) recognized by polyclonal or monoclonal antibodies. After separation by SDS-PAGE, the antigens are electrophoretically transferred onto nitrocellulose membranes. The transferred proteins are bound to the surface of the membrane providing access for immunodetection reagents. All remaining binding sites are blocked by immersing the membrane in a solution containg either a protein or a detergent-based blocking agent. After probing with the primary antibody, the membrane is washed and the antigen is identified by detection with a secondary horseraddish-specific peroxidase-conjugated anti-IgG antibody. Visualization of the antigen/antibody complex is carried out on x-ray film by enhanced chemiluminescence. Experimental terms for the antibodies used are enlisted in Table 2.

Table 2. Experimental terms for the used antibodies.

Antibody, Supplier	Blocking solution	Solution for 1st antibody	Washing buffer system
anti-c-fos; Santa Cruz Biotechnology	Roti Block	5% milk/PBS	PBS/PBST
anti-c-jun; Transduction Laboratories	Roti Block	5% milk/PBS	PBS/PBST
anti-c-src (B12), mouse monoclonal, Santa Cruz Biotechnology	Roti Block	5% milk/PBS	PBS/PBST
anti-EGFR (sc-03), Santa Cruz Biotechnology	Roti Block	5% milk/PBS	PBS/PBST
anti-ERK1/2, Santa Cruz Biotechnology	Roti Block	5% milk/PBS	PBS/PBST
anti-MMP2, Biomol	Roti Block	5% BSA/PBS	PBS/PBST
anti-MMP9, Biomol	Roti Block	5% BSA/PBS	PBS/PBST
anti-MT1-MMP, Chemikon	Roti Block	5% BSA/PBS	PBS/PBST
anti-phosphoEGFR (Y845), Biosource International	5% milk/PBS	5% BSA/PBS	PBS/PBST
anti-phosphoERK, New England Biolabs	Roti Block	5% milk/PBS	PBS/PBST
anti-phosphoJNK, Promega	Roti Block	5% milk/PBS	PBS/PBST
anti-phosphop38, New England Biolabs	5% milk/TBST	5% BSA/TBST	TBST
anti-PI3K p85α, Santa Cruz Biotechnology	Roti Block	5% milk/PBS	PBS/PBST
anti-PI3K p110α, Santa Cruz Biotechnology	Roti Block	5% milk/PBS	PBS/PBST
anti-PI3K p110β, Santa Cruz Biotechnology	Roti Block	5% milk/PBS	PBS/PBST
anti-phosphoSrc (Y418), Biosource International	5% milk/PBS	5% BSA/PBS	PBS/PBST
anti-phosphotyrosine (4G10), Upstate Biotechnology	5% milk/PBS	5% BSA/PBS	PBS/PBST
anti-Pyk2, Transduction Laboratories	Roti Block	5% milk/PBS	PBS/PBST
anti-Ras (pan-ras), Oncogene Research Porducts	Roti Block	5% milk/PBS	PBS/PBST

Stimulation of cells was carried out as indicated in the respective figure legends. In the case of SCLC lines, reactions were stopped by transferring cells on ice. Cells were centrifuged at 1000 rpm and resuspended in 2x SDS-PAGE sample buffer. For all other cell lines, reactions were stopped by washing cells once with ice cold PBS.

Cells were lysed by adding 2x SDS-PAGE sample buffer. Lysates were sonicated and boiled for 3 min at 95°C. Precleared lysates were resolved by SDS-PAGE.
To control the amounts of detected proteins, blots were stripped in stripping buffer for 2 h at 65°C and reprobed with the indicated antibodies.

2x SDS-PAGE sample buffer	5X SDS electrophoresis buffer
125 mM Tris-HCl, pH 6.8	0.125 M Tris base
20% glycerol (v/v)	0.96 M glycine (w/v)
4% SDS (w/v)	0.5% SDS (w/v)
0.2% 2-mercaptoethanol (v/v)	
0.001% bromphenol blue (w/v)	

4x Tris-HCl/SDS pH 6.8
0.5 M Tris base
0.4% SDS (w/v)
adjust to pH 6.8 with HCl

4x Tris-HCl/SDS pH 8.8
1.5 M Tris base
0.4% SDS (w/v)
adjust to pH 8.8 with HCl

Stacking gel
1 ml 30% acrylamide/0.8% bisacrylamide
1.25 ml 4x Tris-HCl/SDS, pH 6.8
3.05 ml ddH_2O
25 µl 10% APS (w/v)
5 µl TEMED

Seperating gel	9%	15%
30% acrylamide/0.8% bisacrylamide	4.50 ml	7.50 ml
4x Tris-HCl/SDS, pH 8.8	3.75 ml	3.75 ml
3.05 ml ddH_2O	6.75 ml	3.75 ml
25 µl 10% APS (w/v)	50 µl	50 µl
5 µl TEMED	10 µl	10 µl

Transfer buffer	Stripping buffer
25 mM Tris-HCl, pH 8.3	100 mM 2-mercaptoethanol
192 mM glycine (w/v)	2.5 mM Tris-HCl, pH 6.7
0.02% SDS (w/v)	2% SDS (w/v)
20% methanol (v/v)	

3.2.2.4. Src kinase activity assay

Src kinase activity assays were performed as previously described (Rodriguez-Fernandez 1996). Cells were lysed in 500 µl ice cold lysis buffer for 15 min. Lysates were cleared by centrifugation, supernatants diluted to 0.5% Triton X-100 with 500 µl HNTG buffer and subjected to immunoprecipitation overnight at 4°C by using a polyclonal anti-Src antibody. 100 µl prewashed protein A-sepharose beads (12.5%, w/v) were added, lysates were incubated at constant rotation for 2 h at 4°C and complexes precipitated by centrifugation. Precipitates were washed three times with ice cold HNTG buffer and subsequently washed twice with Src kinase buffer. After resuspension of the immune complexes in 35 µl Src kinase buffer, Src kinase reactions were performed in the presence of 10 mM $MgCl_2$ and 100 µM Src-peptide (p-Ala-Glu-Glu-Glu-Ile-Tyr-Gly-Glu-Phe-Glu-Ala-Lys-Lys-Lys-Lys-NH_2). Src kinase assay reactions were initiated by addition of 5 µl 100 µM ATP solution containing 2 µCi [γ^{32}-P]ATP. Peptide phosphorylation was carried out for 5 min at 30°C and terminated by the addition of 6 µl 10% phosphoric acid. Reaction mixtures were then spotted onto Whatman P81 chromatography paper squares and washed four times with 150 mM phosphoric acid. Incorporated radioactivity was determined by liquid scintilation spectrometry. All samples were performed in duplicates.

Src kinase-lysis buffer

50 mM Hepes, pH 7.4	50 µM $ZnCl_2$
150 mM NaCl	25 mM NaF
10% glycerol	1 mM Na_3VO_4
1% Triton X-100	1 mM PMSF
1 mM EDTA	10 µg/ml aprotinin
1 mM EGTA	10 µg/ml leupeptin

HNTG buffer

20 mM Hepes, pH 7.5	10 mM NaF
150 mM NaCl	1 mM Na_3VO_4
0.1% Triton X-100	1 mM PMSF
10% glycerol	10 µg/ml aprotinin

Src kinase buffer

50 mM Hepes, pH 7.5
0.1 mM EDTA
0.01% Brij

3.2.2.5. ERK activity assay

Endogenous ERK activity was determined as reported before (Grosse 2000). Cells were lysed in 500 µl ice cold Src kinase-lysis buffer (see above) for 15 min. Lysates were cleared by centrifugation, supernatants diluted to 0.5 % Triton X-100 with 500 µl HNTG buffer (see above) and subjected to immunoprecipitation overnight at 4°C by using a polyclonal anti-ERK2 antibody. 100 µl prewashed protein A-sepharose beads (12.5%, w/v) were added, lysates were incubated at constant rotation for 2 h at 4°C and complexes were precipitated by centrifugation. Precipitates were washed three times with ice cold HNTG buffer and afterwards twice with kinase buffer. Immune complexes were resuspended in 45 µl kinase buffer supplemented with 250 µg/ml myelin basic protein and 2 µM protein kinase A inhibitor, fragment 6-22 amide. The kinase assay reaction was initiated by addition of 5 µl 500 µM ATP solution containing 2 µCi [γ^{32}-P]ATP. The reaction was performed for 30 min at room temperature and terminated by addition of 6 µl 88% formic acid. Reaction mixtures were then spotted onto Whatman P81 chromatography paper squares and washed

four times with 150 mM phosphoric acid. Incorporated radioactivity was determined by liquid scintilation spectrometry. All samples were performed in duplicates.

Kinase buffer

40 mM Hepes, pH 7.5
5 mM magnesium acetate
2 mM dithiothreitol
1 mM EGTA
200 µM Na_3VO_4

3.2.2.6. Ras activation assay

3.2.2.6.1. Preparation of GST fusion proteins containing the Ras-binding domain of Raf-1

GST fusions proteins containing the minimal Ras-binding domain (RBD) of Raf-1 (amino acids 51-131) were prepared as described (Herrmann 1995). Competent *E. coli* cells were transformed with the plasmid pGEX-2T/GST-RBD and were grown to an A_{600} of 0.8. Fusion protein expression was induced by addition of 0.1 mM IPTG. After 2 h, cells were collected by centrifugation at 7700 x g. The pellet was resuspended in 25 ml ice cold PBS. Lysates were sonicated on ice three times for 1 min and Triton X-100 was added to a final concentrtion of 1%. After centrifugation, supernatants were incubated with prewashed glutathione-sepharose 4B for 30 min at room temperature. The fusion protein/sepharose preparation was washed three times in Ras-RIPA buffer and stored at 4°C for up to two weeks.

PBS

136 mM NaCl	0.5 mM $MgCl_2$
2.7 mM KCl	0.5 mM dithiothreitol
8 mM Na_2HPO_4	0.1 µM aprotinin
1.47 mM KH_2PO_4	1 µM leupeptin
1.2 mM $CaCl_2$	1 µM pepstatin A

Ras-RIPA buffer

50 mM Tris-HCl, pH 8.0	0.1% SDS
150 mM NaCl	1 mM PMSF
0.5 mM $MgCl_2$	0.1 µM aprotinin
0.5% deoxycholic acid	1 µM leupeptin
1% Nonidet P - 40	

3.2.2.6.2. Ras activation Assay

Ras activity was determined as described before (de Rooij 1997). After termination of reactions, cells were lysed for 15 min in 500 µl ice cold Ras-RIPA buffer (see above). Lysates were cleared by centrifugation and incubated for 20 min at 4°C with GST-RBD precoupled to glutathione-sepharose 4B. Sepharose beads were collected by centrifugation at 1000 rpm and washed three times with Ras-RIPA buffer on ice. Precipitates were dried up using a hamilton syringe, 2x SDS-PAGE sample buffer was added to the precipitates and GTP-loaded Ras was analysed by SDS-PAGE on 15% gels followed by immunoblotting with an anti-Ras antibody. To demonstrate equal expression levels of Ras, aliquots of the same experiments were subjected to Western blotting. Western blots were probed with anti-Ras antibody.

3.2.2.7. Gelatin zymography

Zymography was performed according to a previously published method (Heussen 1980). Cell culture media were concentrated using a concentration device (Centricon, Millipore, Schwalbach, Germany). Protein contents were determined using the Bardford method. Samples containing 15 µg protein were electrophoresed on 9% non-reducing SDS-PAGE gels containing 0.1% gelatin as a substrate. Gels were washed twice in 2.5% Triton X-100 for 15 min at room temperature. Subsequently, gels were preincubated in reaction buffer for 30 min and incubated in reaction buffer for 18 h at 37°C. Gels were incubated in Coomassie Blue solution for approximately 30 min and afterwards destained in destaining solution. Gelatinolytic activity was visualized as clear areas in a Coomassie Blue-stained gel.

Reaction buffer	Coomassie Blue staining solution
50 mM Tris-HCl, pH 7.6	50% methanol (v/v)
200 mM NaCl	10% acetic acid (v/v)
5 mM $CaCl_2$	40% H_2O (v/v)
1 µM $ZnCl_2$	0.05% Coomassie brilliant blue (w/v)
0.02% Brij-35	

Destaining solution
5% methanol (v/v)
7% acetic acid (v/v)
88% H_2O

3.2.2.8. Colony formation assay

Clonogenic assays were done as described before (Seufferlein 1996). SCLC cells were resuspended in HITESA medium and disaggregated by two passes through a 19-gauge needle. 10^4 cells were mixed with HITESA medium containing 0.3% agarose and galanin or bradykinin in the presence or absence of PP2 and layered over a solid base of 0.5% agarose in HITESA medium with galanin or bradykinin in the presence or absence of PP2 in 6-well plates. The cultures were incubated in a humified atmosphere of 5% CO_2/95% air at 37°C for 21 days and then stained with the vital stain nitro-blue tetrazolium. Colonies of >120 µm in diameter (~16 cells) were counted.

3.2.2.9. Liquid growth assay

Cells were resuspended in HITESA medium and disaggregated by two passes through a 19-gauge needle. At the density of 5 x 10^4 cells in 1 ml HITESA, cells were aliquoted in 24-well plates and treated as described in the figure legends. Cells were incubated in a humified atmosphere of 5% CO_2/95% air at 37°C for 21 days. Viable cells were counted after staining with trypan blue.

3.2.2.10. Detection of apoptosis

For morphological analysis, cells were fixed with 3.7% formalin buffered in PBS containing 0.2% Triton X-100 for 1 h and permeabilized for 10 min with 1% Triton X-100 in PBS supplemented with 0.25 µg/ml bisbenzimide (Hoechst 33258). After

washing with distilled water, single cells were analysed by fluorescence microscopy. Fluorescence microscopy was performed using a Polychrome II monochromator and an IMAGO CCD camera (Till Photonics, Martinsried, Germany) coupled to an inverted Zeiss Axiovert 100 microscope. Transfected cells were identified at 470 nm (excitation maximum of GFP) and apoptosis was analysed at 340 nm (excitation maximum of bisbenzimide). The percentage of apoptotic cells was determined for approximately 50 cells in multiple fields in each independent experiment.

3.2.3. Molecular biological methods

3.2.3.1. Standard procedures

Agarose gel electrophoresis	Aaij 1972
DNA sequencing	Sanger 1977
Ethidium bromide staining of DNA	Maniatis, 1982
Preparation of competent *E. coli* cells	Inoue 1990
Ligation of DNA fragments	Maniatis, 1982
Phenol-chloroform extraction of DNA	Maniatis, 1982
Plasmid preparation using DNA-binding columns	Qiagen, 1993
RNA preparation	Maniatis, 1982
Mutagenesis of DNA	Kunkel 1985
Transformation of *E. coli* cells	Cohen 1972

3.2.3.2. Preparation of total RNA from αT3-1 and SCLC cells

To prepare RNA from αT3-1 cells, cells were grown in a culture flask to 80% confluency. Cell culture medium was discarded and cells resuspended in 3 ml ice cold Trizol solution and incubated on ice for 15 min. Approximately 2 x 10^7 SCLC cells were pelleted by centrifugation at 300 x g to prepare RNA. Cells were resuspended in 1 ml ice cold Trizol solution and incubated on ice for 15 min. Afterwards 200 µl chloroform/ml Trizol solution was added, cells were shaked out and centrifuged at high speed for 10 min at 4°C. The organic phase including the protein-containing interphase was discarded. Total RNA was precipitated by adding

0.7-fold 2-propanol to the aqueous phase. After centrifugation at high speed for 30 min at 4°C, the pellet was washed with 70% ethanol. The dried pellet was resuspended in 30 µl DEPC-treated ddH_2O.

3.2.3.3. RT-PCR of Pyk2 in SCLC cells

Total RNA of SCLC cells was reverse transcribed using an oligo-d(T) primer and SuperSript™ reverse transcriptase according to the manufacturer´s instructions. After digestion with RNase H, PCR reactions to amplify Pyk2 were performed using sense primers Pyk2-1fwd, Pyk2-2fwd and antisense primer Pyk2-1rev, Pyk2-2rev, respectively. GAPDH was amplified as a control. The resulting PCR products were analysed on a 1% agarose gel and visualized by ethidium bromide staining and confirmed by DNA sequencing.

3.2.3.4. RT-PCR of guanine nucleotide-releasing factors for Ras

Total RNA of SCLC cells was reverse transcribed using an oligo-d(T) primer and SuperSript™ reverse transcriptase according to the manufacture´s instructions. After digestion with RNase H, PCR reactions were performed using sense primer RasGRF-fwd and antisense primer RasGRF-rev for RasGRF2 or sense primer RasGRP-fwd and antisense primer RasGRP-rev for RasGRP. GAPDH was amplified as a control. The resulting PCR products were analysed on a 1% agarose gel and visualized by ethidium bromide staining. Sequence confirmation as done by DNA sequencing.

3.2.3.5. Molecular cloning strategies

3.2.3.5.1. Cloning of Pyk2 and generation of Pyk2 mutants

Wild type Pyk2 (Pyk2-wt) was cloned into expression vector pcDNA3.1 from human testis. The N-terminus of Pyk2 was amplified by PCR using forward primer Pyk2-29-F and reverse primer Pyk2-2387-B. The C-terminus was amplified by PCR using forward primer Pyk2-1818-F and reverse primer Pyk2-3184-B. Each PCR product was inserted into pcDNA3.1 by TOPO TA cloning. To generate full-length Pyk2-wt, both constructs were digested with BsmBI and XbaI, the resulting fragments were ligated and subcloned into pcDNA3.1 by TOPO TA cloning.

To introduce mutation K457A into the Pyk2-wt cDNA, a similar cloning strategy as described above was performed. Primer pairs Pyk2-DN-F/Pyk2-3184-B and Pyk2-DN-B/Pyk2-29-F were utilized to generate PCR products from the N- and C-termini of Pyk2. Pyk2 mutants Pyk2-Y402F, Pyk2-P717A and Pyk2-P859A were produced using the QuikChange® multi site-directed mutagenesis kit with mutagenesis primer pairs Pyk2mut-402fwd/Pyk2mut-402-rev, Pyk2mut-717fwd/Pyk2mut-717rev and Pyk2mut-859fwd/Pyk2mut-859rev, respectively. All constructs were confirmed by DNA sequencing.

3.2.3.5.2. Cloning of Pyk2 into the retroviral pLXSN vector

To generate recombinant retroviruses, BamHI-XhoI fragments of pcDNA3.1-Pyk2-wt, pcDNA3.1-Pyk2-Y402F and pcDNA3.1-Pyk2-PKM cDNAs were inserted into the pSV control vector. EcoRI-XhoI fragments of these constructs were cloned into the pLXSN vector. All constructs were confirmed by DNA sequencing.

3.2.4. Reproducibility of results

All assays were performed independently at least three times. One representative experiment is shown.

4. RESULTS

PKC and $[Ca^{2+}]_i$ comprise the two major second messengers in neuropeptide-mediated mitogenic signal transduction. Neuropeptides bind to their cognate receptors to stimulate PLCβ, thereby activating PKC as well as mobilizing $[Ca^{2+}]_i$. In this work mechanisms underlying control of cell proliferation induced by neuropeptides acting on $G_{q/11}$-coupled receptors were investigated. By means of GnRH in gonadotropic αT3-1 cells and of galanin and bradykinin in small cell lung cancer cells, differences could be revealed between Ca^{2+}- and PKC-dependent signaling pathways.

4.1. The gonadotropin-releasing hormone receptor utilizes PKC to activate the ERK/MAPK cascade

4.1.1. GnRH-induced ERK activation depends on gelatinase activity in gonadotropic cells.

The proteolytic production of EGF-like agonists by metalloproteases has recently been identified as the unifying principle governing EGFR transactivation in various cell systems (Prenzel 1999). To identify distinct proteolytic enzymes involved in GnRH-mediated signal transduction, we first chose a pharmacological approach. Since members of the gelatinase subfamily of MMPs (MMP2 and MMP9) are expressed in pituitary tumors and normal pituitary glands (Beaulieu 1999, Paez-Pereda 2000, Turner 2000), we initially focussed on a potential involvement of MMP2 and MMP9 in GnRH-dependent ERK activation in gonadotropes.

Prior to GnRH challenge, αT3-1 cells were incubated with Ro28-2653 (5-biphenyl-4-yl-5-[4-(4-nitro-phenyl)-piperazin-1-yl]pyrimidine-2,4,6-trione), a novel pyrimidine-2,4,6-trion-based MMP inhibitor characterized by a high selectivity for MMP2 and MMP9 (Arlt 2002, Grams 2001, Lein 2002, Table 3) or the broad-spectrum MMP inhibitor batimastat (BB-94, [4-(N-hydroxyamino)-2R-isobutyl-3S-(thiopen-2-ylthiomethyl)-succinyl]-L-phenylalanine-N-methyl-amide). Activated ERK1 and ERK2 were detected by Western blotting with a phosphorylation-specific anti-p42/p44 (pERK1/pERK2) antibody (Figure 6 A). GnRH-elicited ERK activation was almost completely blocked after pretreatment of cells with Ro28-2653 implicating a role for gelatinases in the cross-talk between GnRHR and EGFR, while batimastat had no inhibitory consequence in the concentration tested (10 µM) (Fig. 6 A). Ro28-2653- or

BB-94 treatment of unstimulated cells did not influence the basal phosphorylation status of ERKs (Fig. 6 A).

Table 3. In vitro efficacy of Ro28-2653, batimastat and Ro32-7315.

		Ro28-2653	Batimastat	Ro32-7315
MMP	Common name	IC_{50} [nM]	IC_{50} [nM]	IC_{50} [nM]
MMP1	interstitial collagenase, collagenase 1	16000	25	500
MMP2	gelatinase A	12	32	250
MMP3	stromelysin 1	1800	67	190
MMP8	neutrophil collagenase, collagenase 2	21	27	25
MMP9	gelatinase B	16	23	100
MMP12	human macrophage elastase	9	nd	11
MMP14	MT1-MMP	10	19	nd
ADAM17	TACE	>20000	nd	5

Cited from Beck 2002, Grams 2001, *In vitro* efficacies were determined by a modified fluorescence assay. nd: not determined

Batimastat has been reported to ablate bombesin-induced EGFR transactivation in PC-3 prostate cancer cells (Prenzel 1999). To control for the activity of the batimastat solution used, PC-3 cells were pretreated with batimastat and subsequently stimulated with bombesin. Bombesin-mediated EGFR and ERK activation was detected by Western blotting using phosphorylation-specific antibodies (Fig. 6 B, C). Batimastat abrogated bombesin-induced EGFR- (Fig. 6 B) and ERK phosphorylation (Fig. 6 C) demonstrating that the observed weak inhibitory property of batimastat (Fig. 6 A) was a characteristic feature of αT3-1 cells. Ro28-2653 also harbored inhibitory properties concerning bombesin-mediated EGFR- and ERK phosphorylation in PC-3 cells. These results indicate a predominant role of gelatinases for GPCR-induced ERK phosphorylation in gonadotropic cells.

According to our previous findings in transfected COS-7 cells that stimulation of ERK activity by GnRH is mediated by PKC, short-term TPA challenge of αT3-1 cells resulted in ERK phosphorylation (Fig. 6 D). TPA-elicited ERK-activation was reduced to basal levels in Ro28-2653-treated cells (Fig. 6 D). Incubation of cells with batimastat did not yield an inhibitory effect on TPA-induced ERK phosphorylation

(Fig. 6 D). However, inhibition of EGFR tyrosine kinase activity by the specific tyrphostin AG1478 suppressed ERK activation (Fig. 6 D).

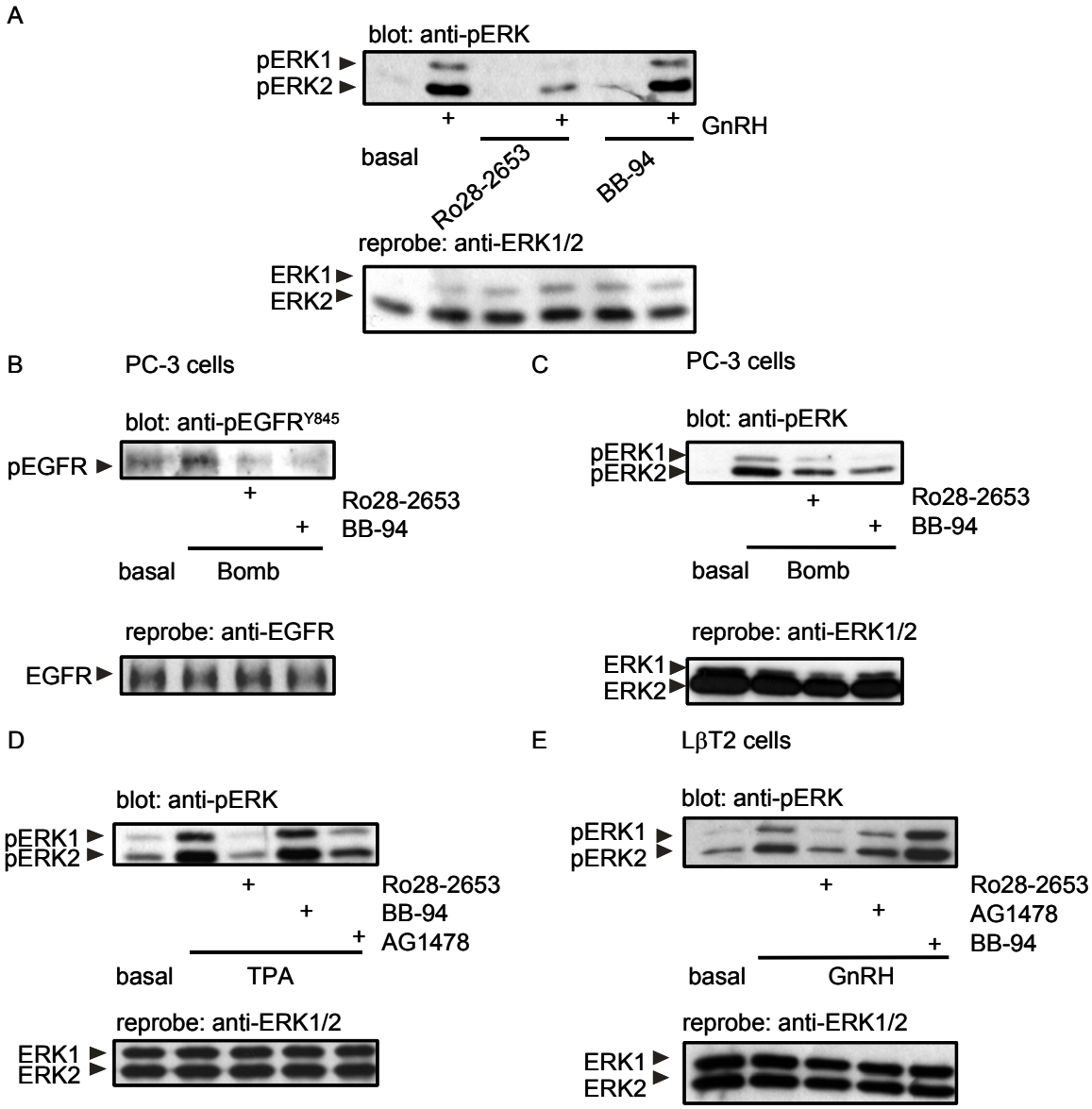


Figure 6. GnRH-induced ERK activation in gonadotropic cells depends on gelatinase activity.

A: *Upper panel,* Serum-starved αT3-1 cells were pretreated with 10 µM Ro28-2653 or 10 µM batimastat (BB-94) for 30 min and stimulated with 1 µM GnRH for 5 min. Western blots of cell lysates were probed with an anti-phosphoERK antibody. *Lower panel*, Blots were reprobed with an anti-ERK1/2 antibody. **B, C:** *Upper panels,* Serum-starved PC-3 cells were preincubated with 10 µM Ro28-2653 or 10 µM batimastat (BB-94) for 30 min and subsequently stimulated with 1 µM bombesin (Bomb) for 5 min. Western blots of cell lysates were probed with an anti-phosphoEGFR (B) or an anti-phosphoERK antibody (C). *Lower panels,* Blots were reprobed with an anti-EGFR (B) or an anti-ERK1/2 antibody (C). **D:** *Upper panel,* Serum-starved αT3-1 cells were pretreated with 10 µM Ro28-2653 or 10 µM batimastat (BB-94) for 30 min and stimulated with 1 µM TPA for 5 min. Western blots of cell lysates were probed with an anti-phosphoERK antibody. *Lower panel*, Blots were reprobed

with an anti-ERK1/2 antibody. **E:** *Upper panel,* Serum-starved LδT2 cells were preincubated with 10 µM Ro28-2653, 10 µM batimastat (BB-94) or 100 nM AG1478 for 30 min and subsequently stimulated with 1 µM GnRH for 5 min. Western blots of cell lysates were probed with an anti-phosphoERK antibody. *Lower panel,* Blots were reprobed with an anti-ERK1/2 antibody.

We further extended our study on another gonadotropic cell line, LβT2 cells. LβT2 cells express both the α and β subunit of LH as well as GnRHR. LβT2 cells probably arose later in ontogeny than αT3-1 cells and thus represent a more mature gonadotropic cell modell (Turgeon 1996). LβT2 cells were pretreated with the gelatinase inhibitor Ro28-2653, batimastat or AG1478 prior to GnRH treatment and stimulation of ERK activity was analysed with an antibody recognizing phosphorylation of ERK1 and ERK2 (Fig. 6 E). In LβT2 cells, GnRH-elicited ERK phosphorylation was blocked to basal levels following pretreatment with Ro28-2653 as well as AG1478, whereas batimastat treatment did not have a bearing on GnRH-mediated ERK phosphorylation (Fig. 6 E).

To address the conundrum as to why batimastat at 10 µM had hardly any effect on $G_{q/11}$-mediated ERK phosphorylation in αT3-1 and LβT2 cells (Fig. 6 A, D, E), while completely abolishing bombesin-dependent EGFR transactivation and ERK phosphorylation in PC-3 cells (Fig. 6 B, C), concentration-response experiments for the two inhibitors, batimastat and Ro28-2653, with concentrations ranging from 0.1 – 50 µM were carried out. Phosphorylation of ERK2 was quantified by densitometric scanning of the x-ray films. GnRH-mediated ERK2 phosphorylation without any inhibition was set as 100%. Data of at least three independent experiments were pooled. As shown in Fig. 6 F, batimastat treatment of αT3-1 cells with increasing concentrations up to 10 µM resulted only in an 18% ± 8.6% suppression of ERK2 phosphorylation. Because of the poor water solubility of batimastat, the highest concentration tested was 50 µM. Even at such a high concentration, ERK2 phosphorylation still amounted to 54% ± 16.6% of the reference value obtained in the absense of batimastat (Fig. 6 G). On the contrary, pretreatment of αT3-1 cells with Ro28-2653 brought about a concentration-dependent decrease of GnRH-mediated ERK2 phosphorylation (Fig. 6 H). At 10 µM Ro28-2653 suppressed $G_{q/11}$-dependent ERK2 activation by more than 70%. At higher inhibitor concentrations (up to 50 µM), ERK2 activation was reduced to about 10% of the initial control value (Fig. 6 I). The enhanced potency of Ro28-2653 compared to batimastat supported the concept that gelatinases represent the main mediators of GnRH-induced signal transduction in

αT3-1 cells, while additional MMPs besides MMP2 and 9 appear to be involved in PC-3 cells.

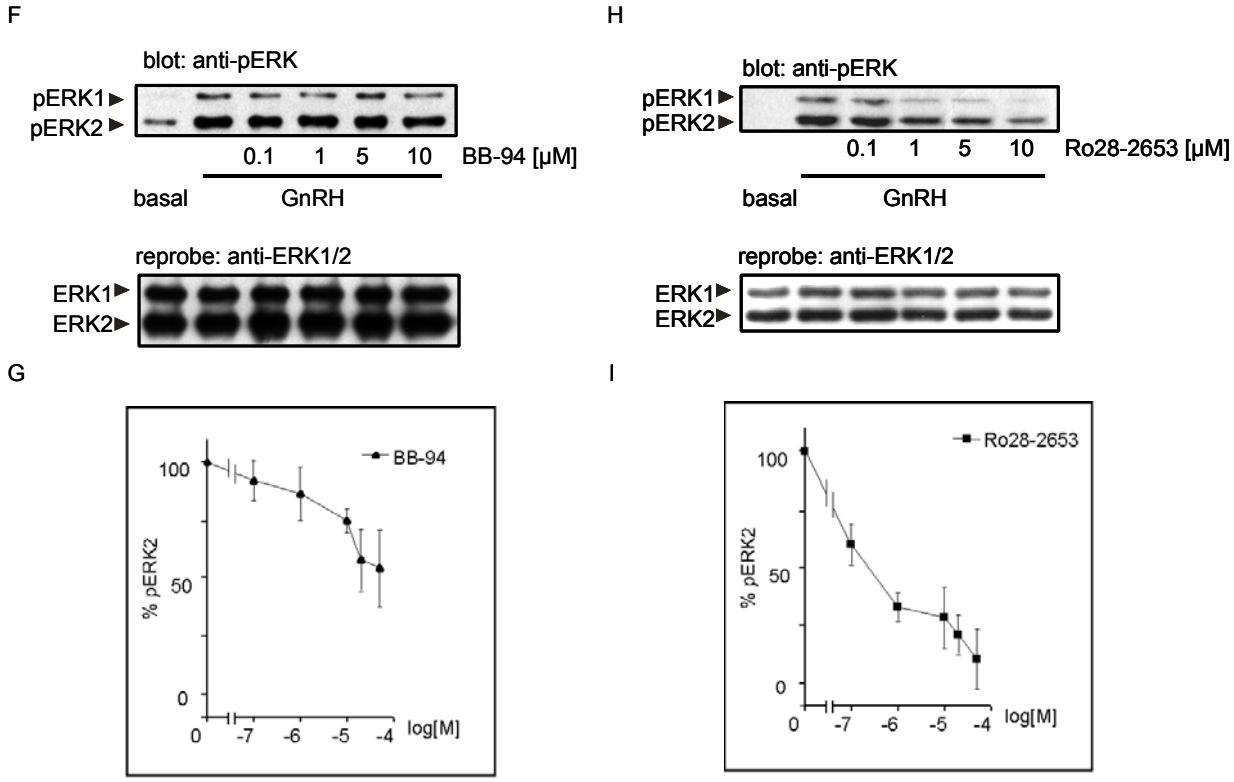


Figure 6. F, H: *Upper panels*, Serum-starved αT3-1 cells were pretreated with the indicated concentrations of batimastat (BB-94) (F) or Ro28-2653 (H) for 30 min and were subsequently stimulated with 1 μM GnRH for 5 min. Western blots of cell lysates were probed with an anti-phosphoERK antibody. *Lower panels*, Blots were reprobed with an anti-ERK1/2 antibody. **G, I:** Serum-starved αT3-1 cells were pretreated with the indicated concentrations of batimastat (BB-94) (G) or Ro28-2653 (I) for 30 min and were subsequently stimulated with 1 μM GnRH for 5 min. Quantification of ERK2 phosphorylation was carried out by densitometry. Pooling of data was achieved by normalizing results to GnRH-mediated ERK2 phosphorylation without any inhibition (100%). Data shown are mean ± SEM of at least three independent experiments.

To unequivocally define the role of gelatinases for the stimulation of ERK activity by GnRH, an independent and more specific ribozyme-based approach was applied (Aigner 2002). The efficiency of the transfection reagent jetPEI to transiently transfect αT3-1 cells was approximately 30 - 50% as assessed by fluorescence microscopy using enhanced green flourescent protein as a marker. Incubation of αT3-1 cells with the transfection preparation entailed no adverse effects on cell proliferation or morphology.

Beforehand, the kinetics and concentration-response of ribozyme-induced downregulation of MMP2 and MMP9 expression in αT3-1 cells were tested. To evaluate the amount of ribozyme needed to suppress GnRH-mediated ERK activation, αT3-1 cells were transfected with increasing amounts of ribozyme Rz-MMP2 directed against MMP2 for an initial time of 6 h and ERK phosphorylation was measured after GnRH challenge as described above. As can be seen in Fig. 7 A, cells transfected with 0.5 µg/well Rz-MMP2 showed the most prominent decline in ERK phosphorylation (Fig. 7 A).

A

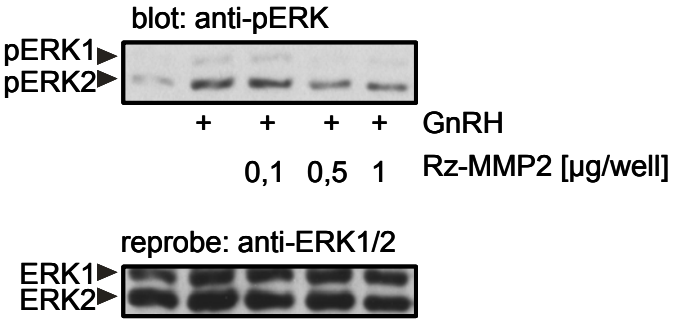


Figure 7. GnRH-induced ERK activation in gonadotropic cells can be inhibited by ribozymes targeting MMP2 or MMP9.

A: *Upper panel*, αT3-1 cells were transiently transfected with 0.1, 0.5 or 1 µg Rz-MMP2 for 6 h. After serum-depletion, cells were stimulated with GnRH for 5 min. Western blots of cell lysates were probed with an anti-phosphoERK antibody. *Lower panel,* Blots were reprobed with an anti-ERK1/2 antibody.

In the kinetic studies, ribozyme targeting of MMP12, human macrophage elastase belonging to the MMP subgroup of stromelysins, was included since Ro28-2653 inhibits MMP12 apart from MMP2 and MMP9 (Grams 2001, Table 3). Cells were incubated with ribozymes Rz-MMP2, Rz-MMP9 or Rz-MMP12 for 6 h, 12 h, 18 h and 24 h, and agonist-induced ERK activation was monitored (Fig. 7 B - E). Incubation of αT3-1 cells with ribozymes MMP2 or MMP9 for 6 h resulted in maximal suppression of GnRH-mediated ERK phosphorylation (Fig. 7 B) while longer incubation times had no further inhibitory effect (Fig. 7 C, D, E). Transfection of Rz-MMP12 did not yield any inhibition during the observation period. Basal values did not change significantly in cells transfected with either ribozyme compared to non-transfected cells (Fig. 7). In conclusion of the data obtained, ribozyme transfection was carried out using 0.5 µg/well for 6 h.

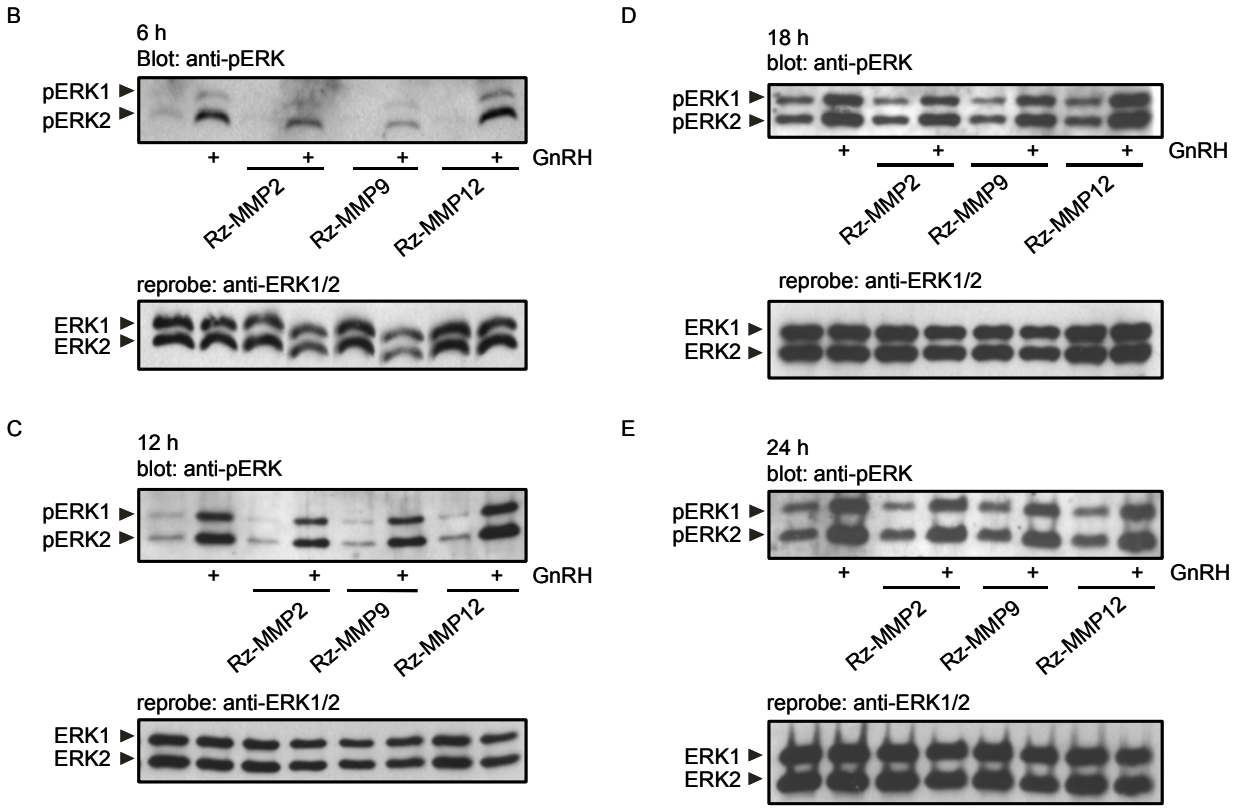


Figure 7. B, C, D, E: *Upper panels*, αT3-1 cells were transiently transfected with ribozymes directed against MMP2 (Rz-MMP2), MMP9 (Rz-MMP9) or MMP12 (Rz-MMP12) for the indicated time periods. Serum-depleted cells were stimulated with 1 µM GnRH for 5 min. Western blots of cell lysates were probed with an anti-phosphoERK antibody. *Lower panels,* Blots were reprobed with an anti-ERK1/2 antibody.

αT3-1 cells were transiently transfected with ribozymes directed against MMP2 (Rz-MMP2), MMP9 (Rz-MMP9), and as controls against MMP12 (Rz-MMP12) or an unrelated fibroblast growth factor-binding protein (Rz-FGF-BP) and Western blots of cell lysates were probed with anti-pERK specific antibodies (Fig. 7 F). Basal values did not change significantly in cells transfected with either ribozyme compared to non-transfected cells (Fig. 7 F). In light of this finding and the previously observation shown in Fig. 6 A that basal values of ERK phosphorylation are not influenced by Ro28-2653- or batimastat pretreatment, inhibitor-treated unstimulated cells were not further included in our study. Cells transfected with Rz-MMP2 or Rz-MMP9 displayed a significant decline in GnRH-induced ERK phosphorylation, whereas transient expression of Rz-MMP12 or the unrelated Rz-FGF-BP did not interfere with GnRH-dependent ERK activation. In summary, these data provide strong support for the concept that GnRH-mediated ERK activation is due to gelatinase-sensitive EGFR transactivation in gonadotropic cells.

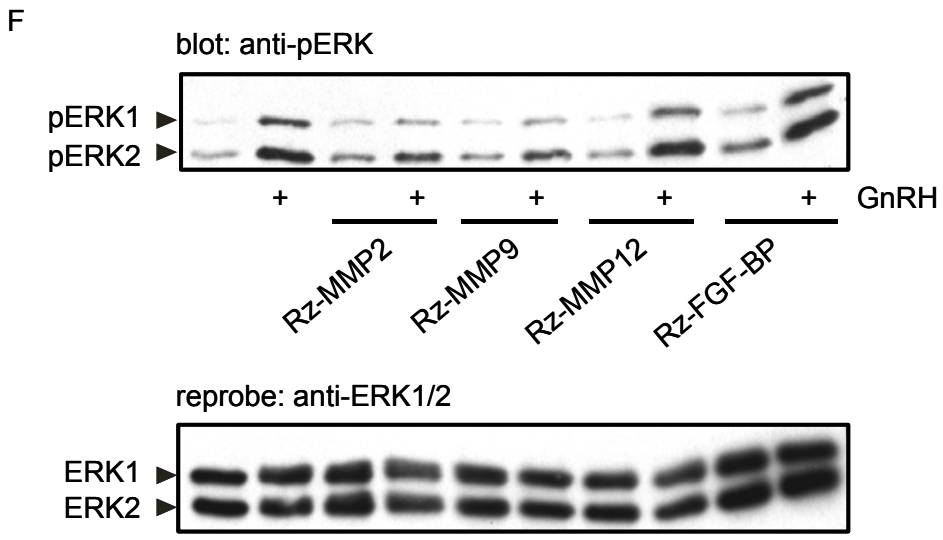


Figure 7. F: *Upper panel*, αT3-1 cells were transiently transfected with ribozymes directed against MMP2 (Rz-MMP2), MMP9 (Rz-MMP9), MMP12 (Rz-MMP12) or FGF-BP (Rz-FGF-BP). Serum-depleted cells were stimulated with 1 µM GnRH for 5 min. Western blots of cell lysates were probed with an anti-phosphoERK antibody. *Lower panel,* Blots were reprobed with an anti-ERK1/2 antibody.

4.1.2. HB-EGF shedding is involved in GnRH-mediated ERK activation.

Each of the known EGF-like mammalian gene products is synthesized as a transmembrane precursor protein subject to proteolytic cleavage of its ectodomain in order to release a soluble growth factor. So far, amphiregulin, HB-EGF and TGFα have been implicated in EGFR transactivation (Gschwind 2003, Pai 2002, Prenzel 1999, Werneburg 2003). To identify the pertinent ligand involved in EGFR transactivation in gonadotropes, we first chose a pharmacological approach by applying a specific ADAM17/TACE inhibitor, Ro32-7315 ((E)-2(R)-[1(S)-(Hydroxy-carbamoyl)-4-phenyl-3-butenyl]-2'-isobutyl-2'-(methanesulfonyl)-4-methylvalerohydra-zide, Beck 2002, Table 3) in αT3-1 cells. As mentioned above, ADAM17/TACE has been uncovered as being responsible for shedding of proamphiregulin leading to EGFR phosphorylation in squamous cancer cells (Gschwind 2003). Furthermore, TGFα has been shown to be an intermediate in GPCR-mediated EGFR transactivation suggesting ADAM17/TACE as the responsible protease (Pai, 2002, Peschon 1998).

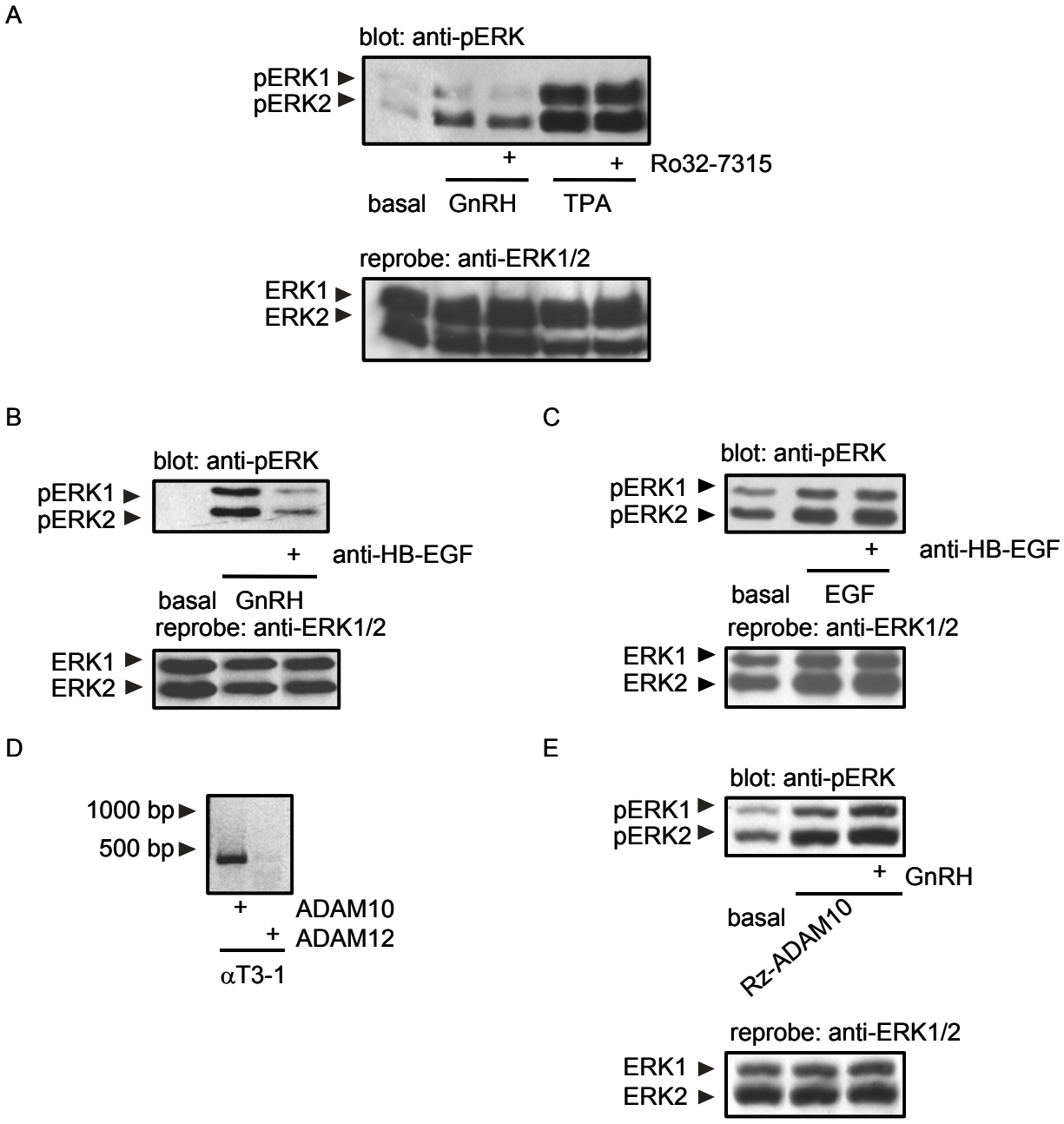


Figure 8. HB-EGF shedding is involved in GnRH-mediated ERK activation in αT3-1 cells.

A: *Upper panel*, Serum-depleted αT3-1 cells were pretreated with 10 µM Ro32-7315 for 30 min prior to stimulation with 1 µM GnRH or 1 µM TPA for 5 min. Western blots of cell lysates were probed with an anti-phosphoERK antibody. *Lower panel*, Blots were reprobed with an antibody detecting ERK1/2. **B:** *Upper panel*, Serum-starved αT3-1 cells were treated with 15 µg/ml anti-HB-EGF antibody for 1 h and subsequently stimulated with 1 µM GnRH for 5 min. Western blots of cell lysates were probed with an anti-phosphoERK antibody. *Lower panel,* Blots were reprobed with an anti-ERK1/2 antibody. **C:** *Upper panel*, Serum-starved αT3-1 cells were treated with 15 µg/ml anti-HB-EGF antibody for 1 h and subsequently stimulated with 10 ng/ml EGF for 5 min. Western blots of cell lysates were probed with an anti-phosphoERK antibody. *Lower panel,* Blots were reprobed with an anti-ERK1/2 antibody. **D:** Total RNA was isolated from αT3-1 cells and RT-PCR was performed using primer pairs for ADAM10 and ADAM12. **E:** *Upper panel*, αT3-1 cells were transiently transfected with Rz-

ADAM10. After serum-starvation, cells were treated with 1 µM GnRH for 5 min. Western blots of cell lysates were probed with an anti-phosphoERK antibody. *Lower panel,* Blots were reprobed with an anti-ERK1/2 antibody.

In Fig. 8 A the effects of Ro32-7315 on GnRH- and TPA-mediated ERK phosphorylation are shown. Phosphorylation of ERKs by both agents did not diminish by Ro32-7315 pretreatment. These data clearly exclude ADAM17/TACE from being involved in GnRH-mediated ERK activation in gonadotropic cells. However, we cannot exclude TGFα or amphiregulin from being the relevant EGFR agonists in αT3-1 cells. Next, we focussed our attention to another EGF-like ligand, HB-EGF. HB-EGF-mediated EGFR transactivation has been reported for numerous GPCRs and cell systems (Prenzel 1999, Uchiyama-Tanaka 2001). To assess the contribution of HB-EGF shedding in GnRH-mediated ERK activation, αT3-1 cells were treated with neutralizing antibodies against HB-EGF. Scavenging of soluble HB-EGF with neutralizing antibodies completely prevented GnRH-elicited ERK activation (Fig. 8 B). On the contrary, ERK phosphorylation induced by exogenously added EGF was not affected by preincubation of cells with anti-HB-EGF (Fig. 8 C).

As mentioned before, two additional disintegrin proteases, ADAM10 and ADAM12, have been shown to participate in RTK transactivation. ADAM12 has been implicated in HB-EGF shedding by vasoactive molecules (Asakura 2002). ADAM10 leads to EGFR transactivation in a variety of different cell systems, however, the precise EGFR agonist was not delineated (Lemjabbar 2002, Yan 2002). To analyse the possible involvement of ADAM10 and ADAM12 in GnRH-mediated EGFR phosphorylation, RT-PCR of total αT3-1 RNA using primer pairs for ADAM10 and ADAM12 were performed. This approach revealed ADAM10 as being expressed in this gonadotropic cell line (Fig. 8 D). αT3-1 cells were transfected with ribozymes targeting ADAM10. After GnRH challenge, Western blots of cell lysates were probed with anti-phosphoERK-specific antibodies. Downregulation of ADAM10 by ribozymes did not have any influence on GnRH-mediated ERK phosphorylation (Fig. 8 E). These data lead to the conclusion that ADAM10 or ADAM17/TACE do not participate in the GnRHR/EGFR cross-talk in αT3-1 cells. In gonadotropic cells, HB-EGF shedding induced by GnRH treatment leads to ERK phosphorylation.

4.1.3. Gelatinase activity is involved in GnRH-mediated EGF receptor transactivation in gonadotropic cells.

To characterize the role of gelatinases in GnRH-initiated signaling events resulting in ERK activation in gonadotropic cells, we first studied the involvement of MMP2 and MMP9 in EGFR transactivation.

For this reason, αT3-1 cells were incubated with Ro28-2653 or batimastat prior to GnRH challenge. Subsequent to agonist stimulation, the EGFR was immunoprecipitated and Western blots of immunoprecipitates were probed with an anti-phosphotyrosine antibody. As shown in Fig. 9 A, GnRH transactivated the EGFR in a Ro28-2653-sensitive manner. The unspecific MMP inhibitor batimastat (BB-94) had only a minor inhibitory effect on GnRH-mediated EGFR activation in αT3-1 cells at the concentration tested (10 µM). As mentioned above, GnRH-induced ERK activation in αT3-1 cells is preceded by EGFR transactivation (Gosse 2000b). Since ERK activation in these cells is dependend on Src activity, we chose antibodies specific for $EGFR^{Y845}$ to detect activated EGFR. Tyrosine 845 within the catalytic domain of the EGFR has been shown to be phosphorylated by Src and is involved in regulating receptor function (Biscardi 1999). GnRH challenge led to phosphorylation of tyrosine 845 of the EGFR which was inhibited by preincubation of the cells with Ro28-2653 (Fig. 9 B). Batimastat treatment had a less pronounced effect (Fig. 9 B). GnRH-induced tyrosine phosphorylation of EGFR was almost completely prevented by pretreatment of cells with 1, 10-phenanthroline (Phe), a chelator of bivalent ions such as zinc, thus lending additional support to the involvement of zinc-dependent endopeptidases.

As a control, the effect of Ro28-2653 and batimastat on EGF-mediated EGFR activation in αT3-1 cells was examined (Fig. 9 C). As expected, EGF increased EGFR phosphorylation in a Ro28-2653-independent way while batimastat treatment showed no inhibition (Fig. 9 C).

In order to strengthen the notion that gelatinases are specifically involved in GnRH-induced EGFR activation in αT3-1 cells, the expression of distinct MMPs was targeted by transfection of ribozymes, and agonist-induced EGFR activation was monitored (Fig. 9 D). The most prominent inhibitory effect was observed when αT3-1 cells were transfected with ribozyme Rz-MMP2. GnRH-mediated EGFR activation was also substantially blocked in cells transfected with ribozymes directed against MMP9. However, GPCR-induced EGFR phosphorylation was nearly unaffected in cells transfected with ribozyme Rz-MMP12. As a negative control, αT3-1 cells were transiently transfected with a ribozyme directed against FGF-BP, because this

binding protein is not involved in the receptor cross-talk under study. As expected, cells transiently expressing Rz-FGF-BP were not affected with regard to GnRH-mediated EGFR tyrosine phosphorylation (Fig. 9 D).

We again extended our study on gonadotropic LβT2 cells. LβT2 cells were pretreated with the gelatinase inhibitor Ro28-2653, batimastat or the EGFR-specific tyrphostin AG1478 prior to GnRH treatment and activation of EGFR was analysed with an antibody recognizing phosphorylation of tyrosine 845 (Fig. 9 E). Pretreatment of cells with Ro28-2653 resulted in a decline of EGFR phosphorylation to basal levels. Further, phosphorylation of EGFR was completely blocked by AG1478 pretreatment. Batimastat did not yield any inhibitory effect on GnRH-elicited EGFR phosphorylation (Fig. 9 E).

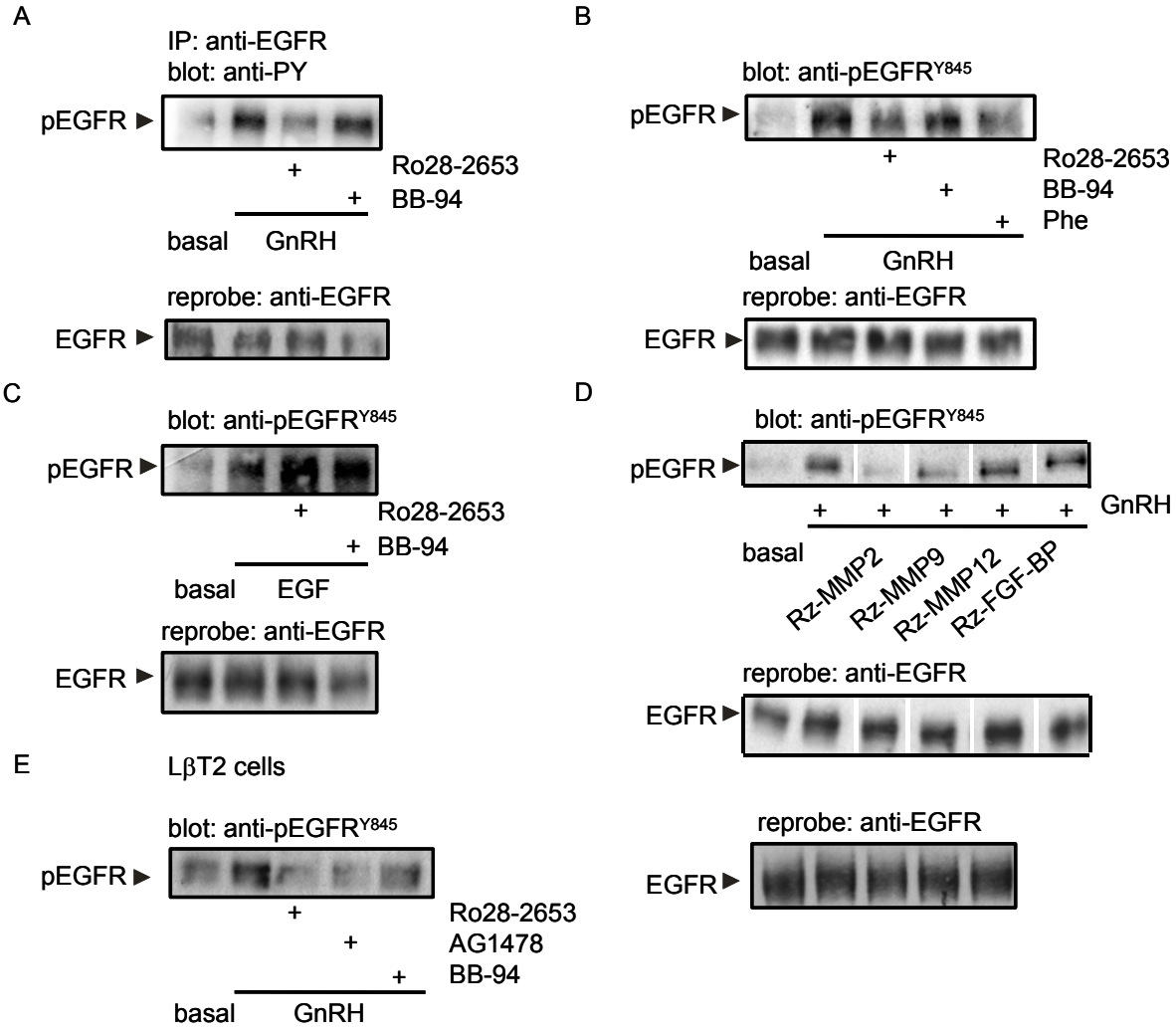


Figure 9. GnRH-induced EGFR transactivation in gonadotropic cells depends on gelatinase activity.

A: *Upper panel,* Serum-starved αT3-1 cells were preincubated with 10 µM Ro28-2653 or 10 µM batimastat (BB-94) for 30 min and subsequently stimulated with 1 µM GnRH for 5 min. After cell lysis, the EGFR was immunoprecipitated using a polyclonal anti-EGFR antibody and

EGFR phosphorylation was determined by an anti-phosphotyrosine (PY)-specific antibody. *Lower panel*, Blots were reprobed with an anti-EGFR antibody. **B:** *Upper panel*, Serum starved αT3-1 cells were preincubated with 10 µM Ro28-2653, 10 µM batimastat (BB-94) or 1, 10-phenanthroline (Phe) for 30 min and subsequently stimulated with 1 µM GnRH for 5 min. Western blots of cell lysates were probed with an anti-phosphoEGFR antibody. *Lower panel,* Blots were reprobed with an anti-EGFR antibody. **C:** *Upper panel,* Serum-starved αT3-1 cells were preincubated with 10 µM Ro28-2653 or 10 µM batimastat (BB-94) for 30 min and subsequently stimulated with 10 ng/ml EGF for 5 min. Western blots of cell lysates were probed with an anti-phosphoEGFR antibody. *Lower panel*, Blots were reprobed with an anti-EGFR antibody. **D:** *Upper panel,* αT3-1 cells were transiently transfected with ribozymes directed against MMP2 (Rz-MMP2), MMP9 (Rz-MMP9), MMP12 (Rz-MMP12) or FGF-BP (Rz-FGF-BP). After serum-depletion, cells were stimulated with 1 µM GnRH for 5 min. Western blots of cell lysates were probed with an anti-phosphoEGFR antibody. *Lower panel,* Blots were reprobed with an anti-EGFR antibody. **E:** *Upper panel,* Serum-starved LβT2 cells were preincubated with 10 µM Ro28-2653, 10 µM batimastat (BB-94) or 100 nM AG1478 for 30 min and then stimulated with 1 µM GnRH for 5 min. Western blots of cell lysates were probed with an anti-phosphoEGFR antibody. *Lower panel,* Blots were reprobed with an anti-EGFR antibody.

To investigate whether gelatinases are activated in a GnRH-dependent manner, conditioned αT3-1 cell culture supernatants were concentrated, resolved by SDS-PAGE and probed with anti-MMP2 or anti-MMP9 antibodies. A 5-min GnRH challenge resulted in enhanced release of MMP2 (Fig. 10 A, B, upper panels). Preincubation of αT3-1 cells with Ro28-2653 decreased MMP2 contents of the medium as did transfection with ribozymes targeting MMP2 (Fig. 10 A, B, upper panels). On the contrary, transfection of cells with Rz-MMP9, Rz-MMP12 or Rz-FGF-BP had no impact on MMP2 levels (Fig. 10 B, upper panel). GnRH stimulation also led to an increase in MMP9 protein levels in the medium (Fig. 10 A, B, lower panels). The release of MMP9 was counteracted by preincubation of cells with Ro28-2653 (Fig. 10 A, lower panel) or transfection with Rz-MMP9 (Fig. 10 B, lower panel). Targeting MMP2 via Rz-MMP2 entailed a decrease in MMP9 release (Fig. 10 B, lower panel) potentially indicating a role for MMP2 in MMP9 activation (Murphy 1999). MMP12 or FGF-BP do not govern MMP9 activation since downregulation of these proteins did not inhibit MMP9 activation (Fig. 10 B, lower panel).

To directly show that the enzymatic activity of gelatinases is subject to hormonal regulation in αT3-1 cells, cell culture media were analysed by gelatin zymography (Fig. 10 C). Under all conditions tested, we observed a gelatinolytic 72-kDa band corresponding to the proform of MMP2. An additional band migrating with a molecular mass of approximately 66 kDa was detected after GnRH stimulation of cells. It represents the active form of MMP2. Activated MMP2 was not demonstrable in conditioned media of cells pretreated with Ro28-2653. A gelatinolytic band with a

molecular mass of approximately 92 kDa corresponding to proMMP9 was noted in all samples. Yet, release of activated MMP9, which has a molecular mass of approximately 68 kDa, subsequent to GnRH treatment could not be readily observed (Fig. 10 C).

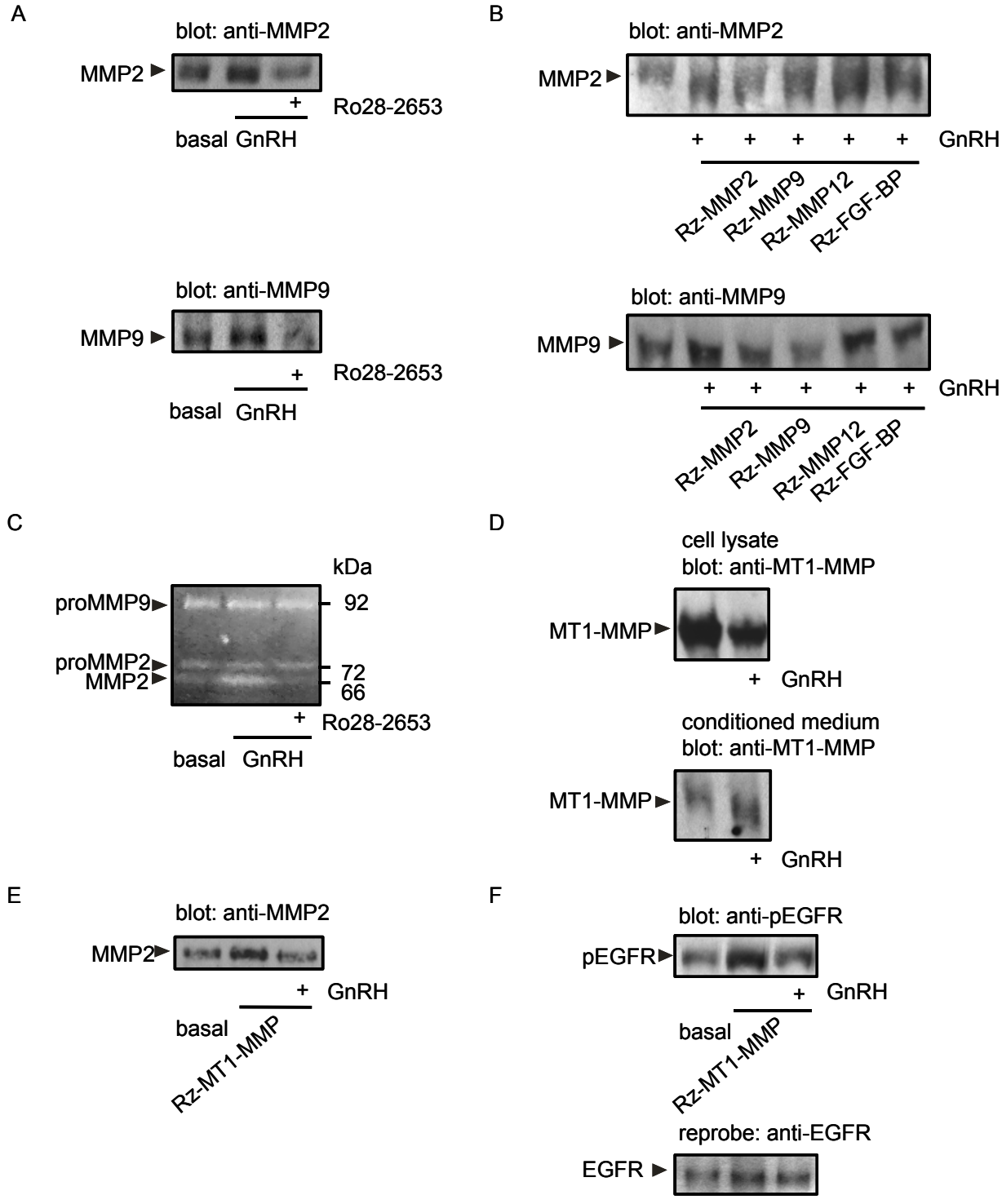


Figure 10. GnRH-mediated gelatinase activation is inhibited by Ro28-2653 as well as targeting gelatinases by ribozymes. MT1-MMP is activated in a GnRH-dependent manner in αT31- cells.

A: Serum-starved αT3-1 cells were preincubated with 10 µM Ro28-2653 for 30 min and subsequently stimulated with 1 µM GnRH for 5 min. Culture media were collected and concentrated. Western blots of samples containing 15 µg protein were probed with an anti-MMP2 antibody (*upper panel*) or an antibody directed against MMP9 (*lower panel*). **B:** αT3-1 cells weretransiently transfected with ribozymes directed against MMP2 (Rz-MMP2), MMP9 (Rz-MMP9), MMP12 (Rz-MMP12) or FGF-BP (Rz-FGF-BP). After serum-depletion, cells were stimulated with 1 µM GnRH for 5 min. Culture media were collected and concentrated. Western blots of samples containing 15 µg protein were probed with an anti-MMP2 (*upper panel*) or an anti-MMP9 (*lower panel*) antibody. **C:** Serum-starved αT3-1 cells were preincubated with 10 µM Ro28-2653 for 30 min and subsequently stimulated with 1 µM GnRH for 5 min. Culture media were collected and concentrated. Samples containing 15 µg protein were subjected to gelatin zymography. Molecular weight markers are indicated at the right. **D:** Serum-starved αT3-1 cells were stimulated with 1 µM GnRH for 5 min. Western blots of cell lysates (*upper panel*) and concentrated culture medium containing 15 µg protein (*lower panel*) were probed with anti-MT1-MMP antibodies. **E:** αT3-1 cells were transfected with ribozymes directed against MT1-MMP (Rz-MT1-MMP). After serum-depletion, cells were stimulated with 1 µM GnRH for 5 min. After concentration of culture media, Western blots of samples containing 15 µg protein were probed with an anti-MMP2 antibody. **F:** *Upper panel*, αT3-1 cells were transiently transfected with ribozyme Rz-MT1-MMP. After serum-starvation, cells were stimulated with 1 µM GnRH for 5 min. Western blots of cell lysates were probed with an anti-phosphoEGFR antibody. *Lower panel,* Blots were reprobed with an anti-EGFR antibody.

MT1-MMP plays a prominent role in the activation of gelatinases. MT1-MMP is anchored to the plasma membrane tethering secreted proMMP2 in a complex also containing TIMP2. As a result of activating stimuli, proMMP2 is cleaved and activated by MT1-MMP (Werb 1997, Westermarck 1999). Along these lines, αT3-1 cells were stimulated with GnRH and MT1-MMP was detected in cell lysates as well as concentrated conditioned medium (Fig. 10 D). As shown in Fig. 10 D, upper panel, a 5-min stimulation of αT3-1 cells with GnRH resulted in a decrease of MT1-MMP in cell lysates, while agonist challenge led to secretion of MT1-MMP into the cell medium (Fig. 10 D, lower panel).

To further show that MT1-MMP is not only rapidly activated by GnRH stimulation but is also responsible for MMP2 activation (Werb 1997), MT1-MMP was targeted with Rz-MT1-MMP and the protein level of MMP2 was detected in conditioned medium of αT3-1 cells 5 min after GnRH challenge (Fig. 10 E). Rz-MT1-MMP transfection reduced GnRH-mediated MMP2 levels in conditioned medium of αT3-1 cells (Fig. 10 E). Finally, the involvement of MT1-MMP in GnRH-mediated EGFR phosphorylation was investigated. αT3-1 cells were transfected with ribozyme Rz-MT1-MMP and the phosphorylation status of EGFR was analysed after GnRH challenge (Fig. 10 F). Induction of EGFR phosphorylation by GnRH was markedly suppressed in cells transfected with Rz-MT1-MMP. In summary, our data show that the cross-talk

between GnRHR and EGFR proceeds via MT1-MMP and gelatinases. Furthermore, we provide evidence that MT1-MMP is involved in MMP2 activation in αT3-1 cells.

4.1.4. HB-EGF shedding mediates EGF receptor phosphorylation in gonadotropic cells.

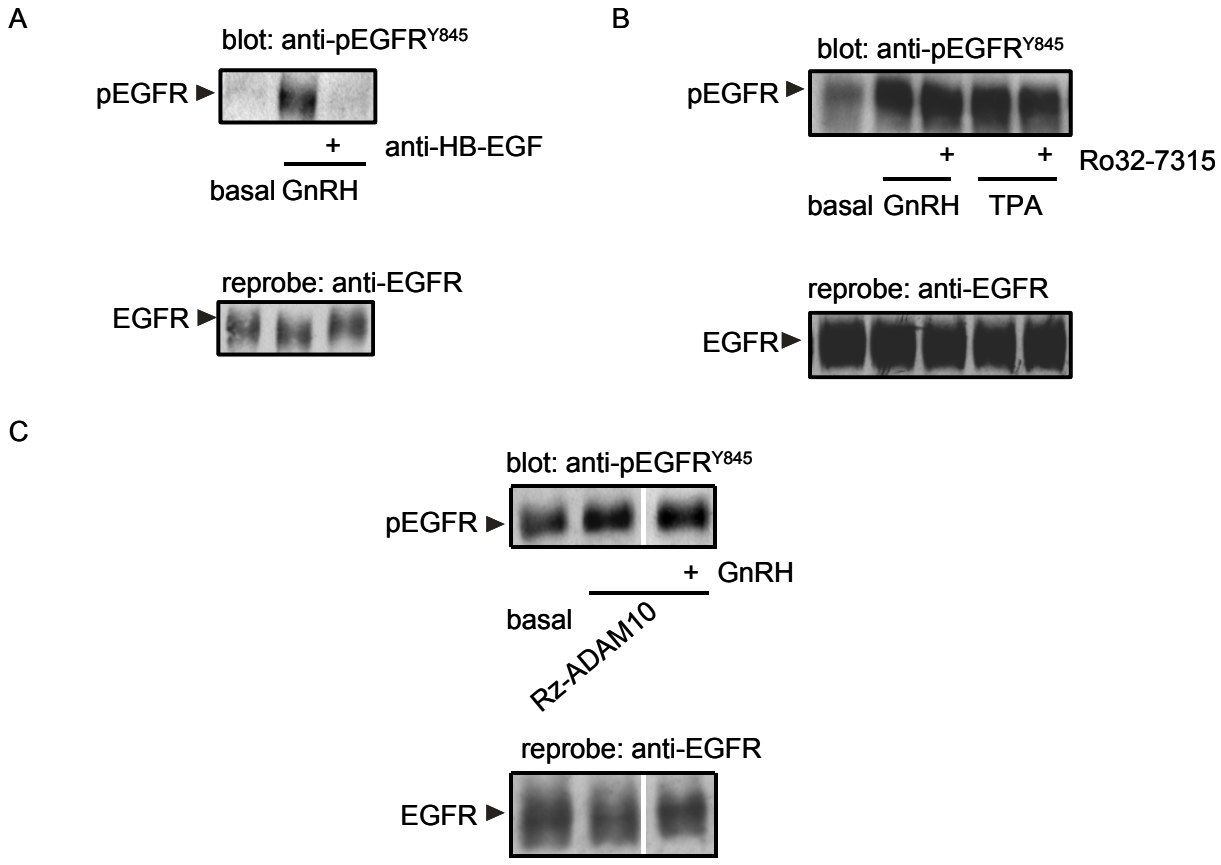


Figure 11. HB-EGF shedding is involved in GnRH-mediated EGFR transactivation in αT3-1 cells.

A: *Upper panel*, Serum-starved αT3-1 cells were treated with 15 µg/ml anti-HB-EGF antibody for 1 h and subsequently stimulated with 1 µM GnRH for 5 min. Western blots of cell lysates were probed with an anti-phosphoEGFR antibody. *Lower panel,* Blots were reprobed with an anti-EGFR antibody. **B:** *Upper panel*, Serum-starved αT3-1 cells were pretreated with 10 µM Ro32-7315 for 30 min prior to stimulation with 1 µM GnRH or 1 µM TPA for 5 min. Western blots of cell lysates were probed with anti-phosphoEGFR antibody. *Lower panel*, Blots were reprobed with antibodies detecting EGFR. **C:** *Upper panel*, αT3-1 cells were transiently transfected with Rz-ADAM10. After serum-starvation, cells were treated with 1 µM GnRH for 5 min. Western blots of cell lysates were probed with an anti-phosphoEGFR antibody. *Lower panel,* Blots were reprobed with an anti-EGFR antibody.

We have shown that HB-EGF is the predominant ligand for transactivation-mediated ERK activation in αT3-1 cells. To test whether liberated HB-EGF serves to induce EGFR activation, lysates of αT3-1 cells preincubated with anti-HB-EGF prior to

GnRH challenge were probed with anti-phosphoEGFR antibodies. In line with our data obtained when monitoring ERK phosphorylation, trapping of HB-EGF strongly inhibited GnRH-mediated EGFR activation (Fig. 11 A). To elucidate the role of ADAM17/TACE-induced shedding in $G_{q/11}$-arbitrated EGFR activation, we applied Ro32-7315 in αT3-1 cells. In Fig. 11 B the effects of Ro32-7315 on GnRH- and TPA-mediated EGFR activation are shown. GnRH transactivates EGFR in a ADAM17/TACE-independent fashion. Furthermore, TPA-elicited EGFR phosphorylation was not inhibited by Ro32-7315 treatment (Fig. 11 B). As performed on the ERK level before (Fig. 8 E), the involvement of ADAM10 in GnRH-induced EGFR phosphorylation was studied by transiently transfecting αT3-1 cells with Rz-ADAM10. Phosphorylated EGFR was detected with an anti-phosphoEGFR^{Y845}-specific antibody (Fig. 11 C). Ribozyme targeting of ADAM10 did not inhibit GnRH-mediated EGFR phosphorylation (Fig. 11 C). In summary, these data elucidate that HB-EGF shedding induced by GnRH treatment leads to EGFR phosphorylation in gonadotropic cells.

4.1.5. GnRH-mediated Src activation in αT3-1 cells depends on gelatinase activity.

We have recently shown that inhibition of Src activity suppresses GnRHR-mediated GTP-loading of Ras and subsequent ERK activation in αT3-1 and transfected COS-7 cells (Grosse 2000b). To directly address the role of Src kinases in GnRH-induced EGFR transactivation in αT3-1 cells, Src kinase activity was blocked by the specific Src inhibitor PP2. As shown in Fig. 12 A, preincubation of cells with PP2 resulted in diminished GnRH-mediated EGFR tyrosine phosphorylation. Likewise, pretreatment of cells with AG1478 abrogated EGFR activation stimulated by GnRH treatment (Fig. 12 A). Furthermore, αT3-1 cells were pretreated with PP2 and AG1478 and activated Src was monitored in cell lysates with an antibody recognizing phosphorylation of tyrosine 424 (418 in human Src) located in the catalytic domain of Src. Phosphorylation of tyrosine 424 is necessary for full catalytic activity and Src^{Y424} has been shown to mediate phosphorylation of tyrosine 845 of the EGFR (Biscardi 1999). GnRH challenge led to increased Src phosphorylation which was not inhibited in cells pretreated with AG1478 whereas PP2 treatment resulted in reduced phosphorylation of Src (Fig. 12 B). Thus, EGFR tyrosine kinase activity is not required for Src phosphorylation at tyrosine 424 and Src appears to be located upstream of the EGFR in the cross-talk between the RTK and the GnRHR.

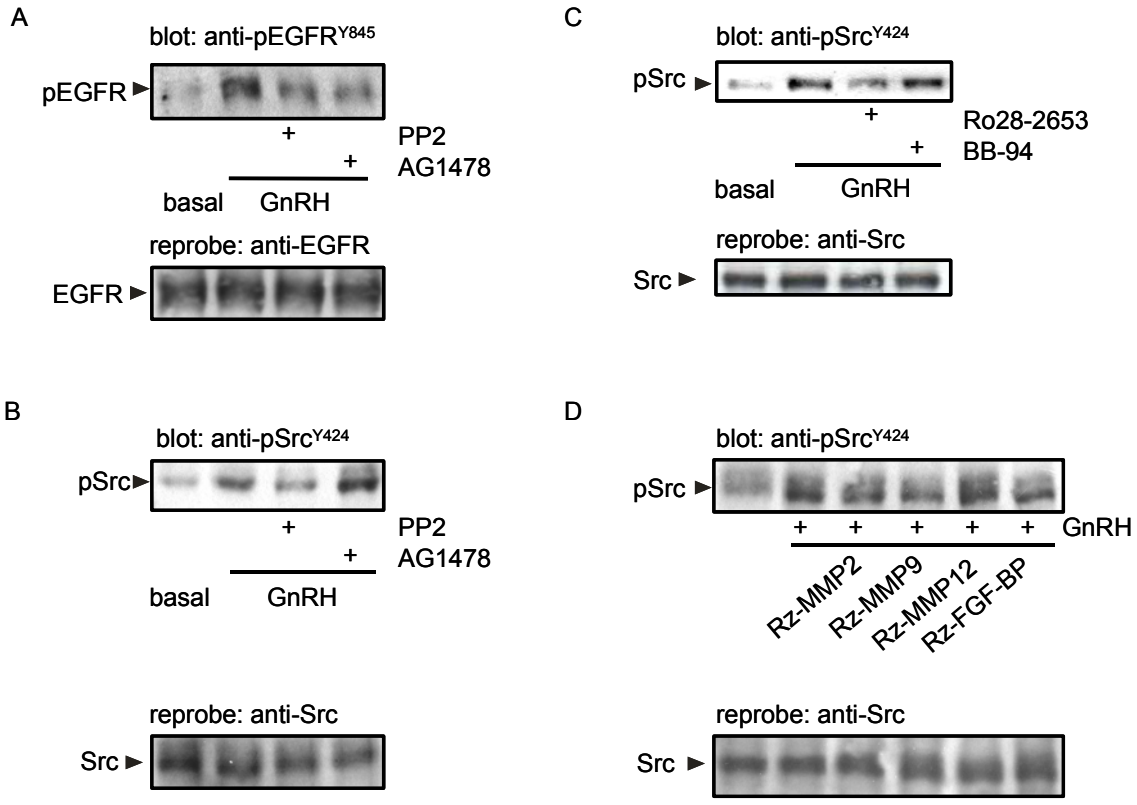


Figure 12. Src is involved in GnRH-mediated EGF receptor transactivation in αT3-1 cells.

A, B: *Upper panels*, Serum-depleted αT3-1 cells were preincubated with 1 µM PP2 or 100 nM AG1478 for 30 min and subsequently stimulated with 1 µM GnRH for 5 min. Western blots of cell lysates were probed with an anti-phosphoEGFR (A) or an anti-phosphoSrc (B) antibody. *Lower panels,* Blots were reprobed with an anti-EGFR (A) or an anti-Src (B) antibody. **C:** *Upper panel*, Serum-starved αT3-1 cells were preincubated with 10 µM Ro28-2653 or 10 µM batimastat (BB-94) for 30 min and subsequently incubated with 1 µM GnRH. Western blots of cell lysates were probed with an anti-phosphoSrc antibody. *Lower panel*, Blots were reprobed with an anti-Src antibody. **D:** *Upper panel,* αT3-1 cells were transiently transfected with ribozymes directed against MMP2 (Rz-MMP2), MMP9 (Rz-MMP9), MMP12 (Rz-MMP12) or FGF-BP (Rz-FGF-BP). After serum-depletion, cells were stimulated with 1 µM GnRH for 5 min. Western blots of cell lysates were probed with an anti-phosphoSrc antibody. *Lower panel,* Blots were reprobed with an anti-Src antibody.

To assess the contribution of MMPs in GnRH-mediated Src phosphorylation, gelatinases were blocked by preincubation with Ro28-2653 prior to GnRH stimulation (Fig. 12 C). GnRH-mediated Src phosphorylation was sensitive to inhibition of MMP2 and MMP9 activities by Ro28-2653 as shown in Fig. 12 C, while batimastat had no inhibitory effect. This pharmacological approach indicates that gelatinases are located upstream of Src in the pathway emanating from GnRH coupling to its

cognate receptor. To further test this concept, MMP2 and MMP9 were downregulated by ribozyme transfection of αT3-1 cells as described above. Transfection with ribozymes against MMP2 or MMP9 confirmed the pharmacological inhibitor studies by suppressing GnRH-mediated Src activation (Fig. 12 D) while Rz-MMP12 or Rz-FGF-BP did not show inhibitory properties. Considering that Src or EGFR blockage did not have any impact on the activation of MMP2 or MMP9 by GnRH (Fig. 12 E), we conclude that Src is interposed between activated gelatinases and the EGFR in αT3-1 cells.

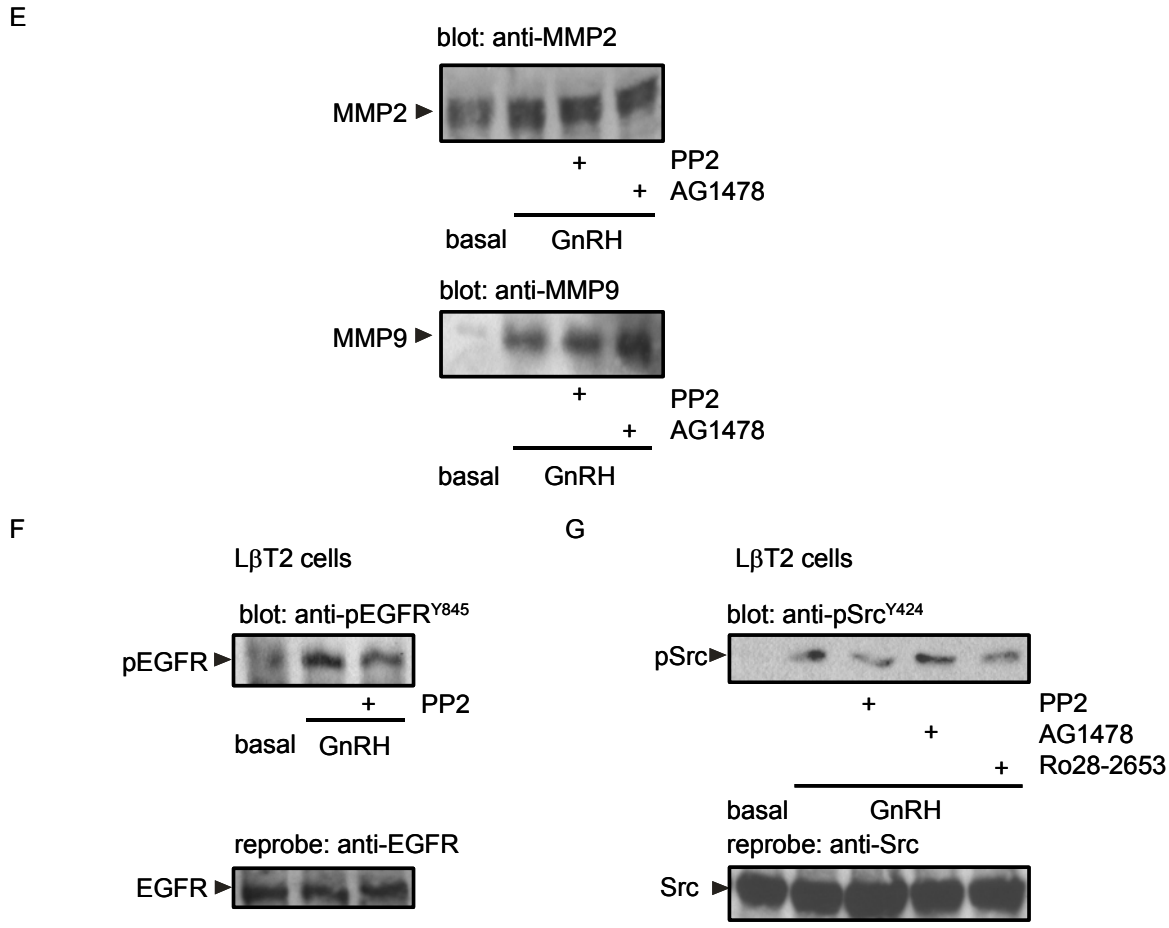


Figure 12. E: Serum-starved αT3-1 cells were pretreated with 1 µM PP2 or 100 nM AG1478 for 30 min and treated with 1 µM GnRH for 5 min. Culture media were collected and concentrated. Western blots of samples containing 15 µg protein were probed with anti-MMP2- (upper panel) or anti-MMP9 (lower panel) antibodies. **F:** *Upper panel*, Serum-depleted LβT2 cells were preincubated with 1 µM PP2 for 30 min and incubated with 1 µM GnRH for 5 min. Western blots of cell lysates were probed with an anti-phosphoEGFR antibody. *Lower panel*, Blots were reprobed with an anti-EGFR antibody. **G:** *Upper panel*, Serum-depleted LβT2 cells were preincubated with 1 µM PP2, 100 nM AG1478 or 100 µM Ro28-2653 for 30 min and incubated with 1 µM GnRH for 5 min. Western blots of cell lysates were probed with an anti-phosphoSrc antibody. *Lower panel*, Blots were reprobed with an anti-Src antibody.

Yet again, we extended our study on LβT2 cells to address the issue of Src localization. LβT2 cells were pretreated with PP2 and EGFR phosphorylation was detected. GnRH transactivated the EGFR in a PP2-dependent manner (Fig. 12 F). In addition, cells were treated with PP2 or AG1478 and Src phosphorylation was detected. Treatment of cells with PP2 led to inhibition of GnRH-mediated Src activation whereas AG1478 showed no inhibitory properties (Fig. 12 G). Gelatinases contribute to GnRH-induced Src phosphorylation since blockage of MMP2 and MMP9 by Ro28-2653 diminished GnRH-mediated Src phosphorylation (Fig. 12 G).

4.1.6. GnRH-elicited Ras activation is dependend on EGF receptor transactivation and gelatinase activity in gonadotropic cells.

Mitogenic signaling of GPCRs is realized via both Ras-dependent and –independent pathways (Gudermann 2000). GTP-loaded Ras subsequently engages the Raf/MEK/ERK cascade indistinguishable from the mechanism initiated by ligand-bound RTKs. Interpretation of Ras activation by G_q-coupled receptors, however, is complicated by the fact that most heptahelical receptors concomitantly couple to an assortment of different G proteins and so GPCR-mediated Ras activation still is a rather contradictory subject (Gudermann 2000). We have shown recently that ERK activation in gonadotropic αT3-1 cells is accomplished via activation of Ras (Grosse 2000b). To study the contribution of EGFR activation on GnRH-mediated GTP-loading of Ras, αT3-1 cells were preincubated with AG1478 and subsequently treated with GnRH or TPA. A GST-fusion protein containing the minimal Ras-binding domain of Raf-1 was used to precipitate the activated form of Ras. GnRH challenge led to GTP-loading of Ras which was reduced substantially by preincubation of cells with AG1478 (Fig. 13 A). GnRH signaling to ERK can be fully mimicked by PKC activation induced by short-term TPA treatment (Fig. 13 A). TPA-elicited Ras activity was also substantially diminished by AG1478 pretreatment (Fig. 13 A). To define the roles of PKC and Src in GnRH-mediated Ras activation, both kinases were inhibited by specific inhibitors GF109203X or PP2, respectively (Fig. 13 B). As is clearly visible in Fig. 13 B, GnRH-mediated GTP-loading of Ras is attenuated by pretreatment of cells with GF109203X or PP2. To test whether gelatinase-mediated EGFR transactivation is a prerequisite for Ras activation by GnRH, αT3-1 cells were pretreated with Ro28-2653 or batimastat prior to GnRH or short-term phorbol ester treatment. Preincubation of cells with Ro28-2653 reduced the amounts of precipitated Ras proteins induced by both stimuli (Fig. 13 C, D). On the contrary,

pretreatment of cells with batimastat did not adversely affect GnRH- or TPA-mediated activation of Ras. To summarize, our data demonstrate that MMP2 and MMP9 mediate GnRH activation of EGFR resulting in the engagement of a PKC- and Src-dependent Ras signaling pathway in αT3-1 cells.

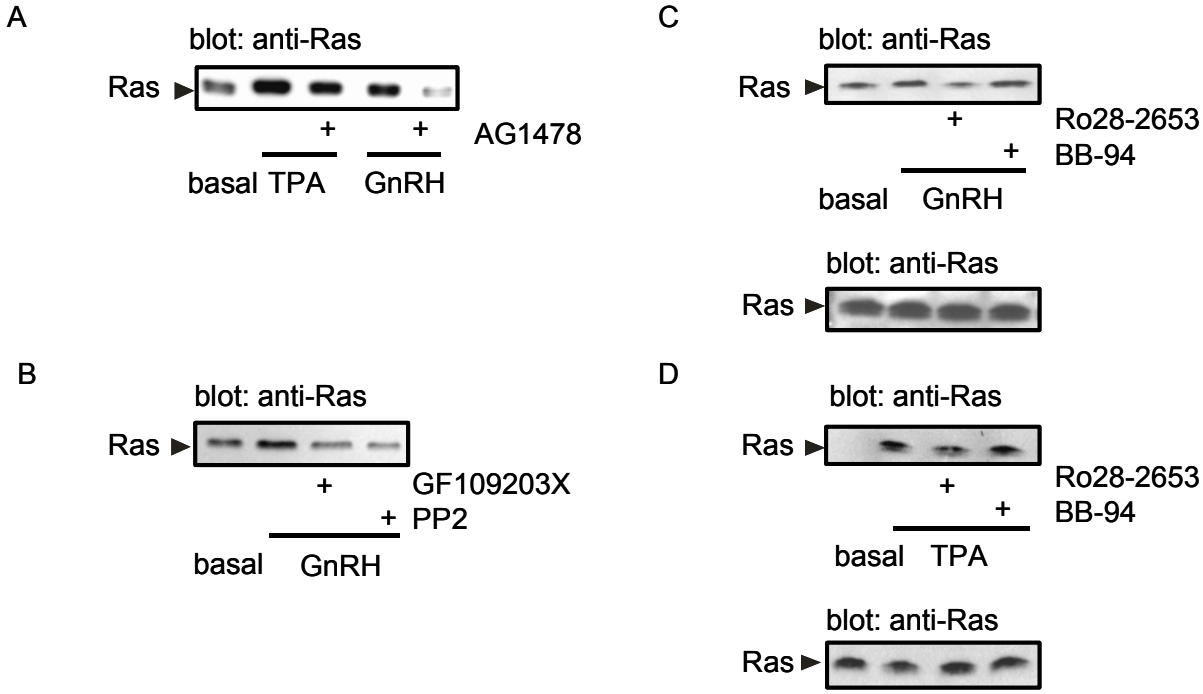


Figure 13. $G_{q/11}$-mediated Ras activation involves MMP2 and MMP9 activities.

A: Serum-starved αT3-1 cells were incubated with 200 nM GnRH or 100 ng/ml TPA for 5 min after pretreatment with 100 nM AG1478 for 20 min. Cell lysates were incubated with GST-RBD precoupled to glutathione-sepharose at 4°C for 30 min. GTP-loaded Ras was analysed by SDS-PAGE followed by immunoblotting with an anti-Ras antibody. **B:** Serum-starved αT3-1 cells were preincubated with 1 µM GF109203X or 1 µM PP2 for 20 min prior to 200 nM GnRH challenge. Cell lysates were incubated with GST-RBD precoupled to glutathione-sepharose at 4°C for 30 min. GTP-loaded Ras was analysed by SDS-PAGE followed by immunoblotting with an anti-Ras antibody. **C, D:** *Upper panels*, Serum-depleted αT3-1 cells were preincubated with 10 µM Ro28-2653 or 10 µM batimastat (BB-94) for 30 min and then stimulated with 1 µM GnRH (C) or 1 µM TPA (D) for 5 min. Cell lysates were incubated with GST-RBD precoupled to glutathione-sepharose at 4°C for 30 min. GTP-loaded Ras was analysed by SDS-PAGE followed by immunoblotting with an anti-ras antibody. *Lower panels*, Aliquots of the used lysates were subjected to SDS-PAGE followed by immunoblotting with an anti-Ras antibody.

4.1.7. EGF receptor tyrosine kinase and MMP activities are not required to mediate GnRH-induced activation of JNK and p38MAPK.

p38MAPK has been implicated in being regulated by EGFR transactivation in a variety of different cell systems whereas GPCR/EGFR cross-talk has not been

reported to play a role in JNK activation (Eguchi 2001, Kanda 2001). Apart from engaging the ERK cascade, GnRH also stimulates the activities of all three stress-related MAPKs (Naor 2000). We have previously shown that inhibition of EGFR by AG1478 retarded the onset of agonist-dependent ERK activation in αT3-1 cells (Grosse 2000b). To extend this experimental approach to the JNK and p38MAPK cascades, we monitored the time course of JNK and p38MAPK activation in αT3-1 cells. Cells were pretreated with AG1478, Ro28-2653 or batimastat and subsequently stimulated with GnRH for multiple time points ranging from 5 min to 1 h. Western blots of cell lysates were performed with antibodies directed against phosphorylated JNK1/2 and p38MAPK (Fig. 14).

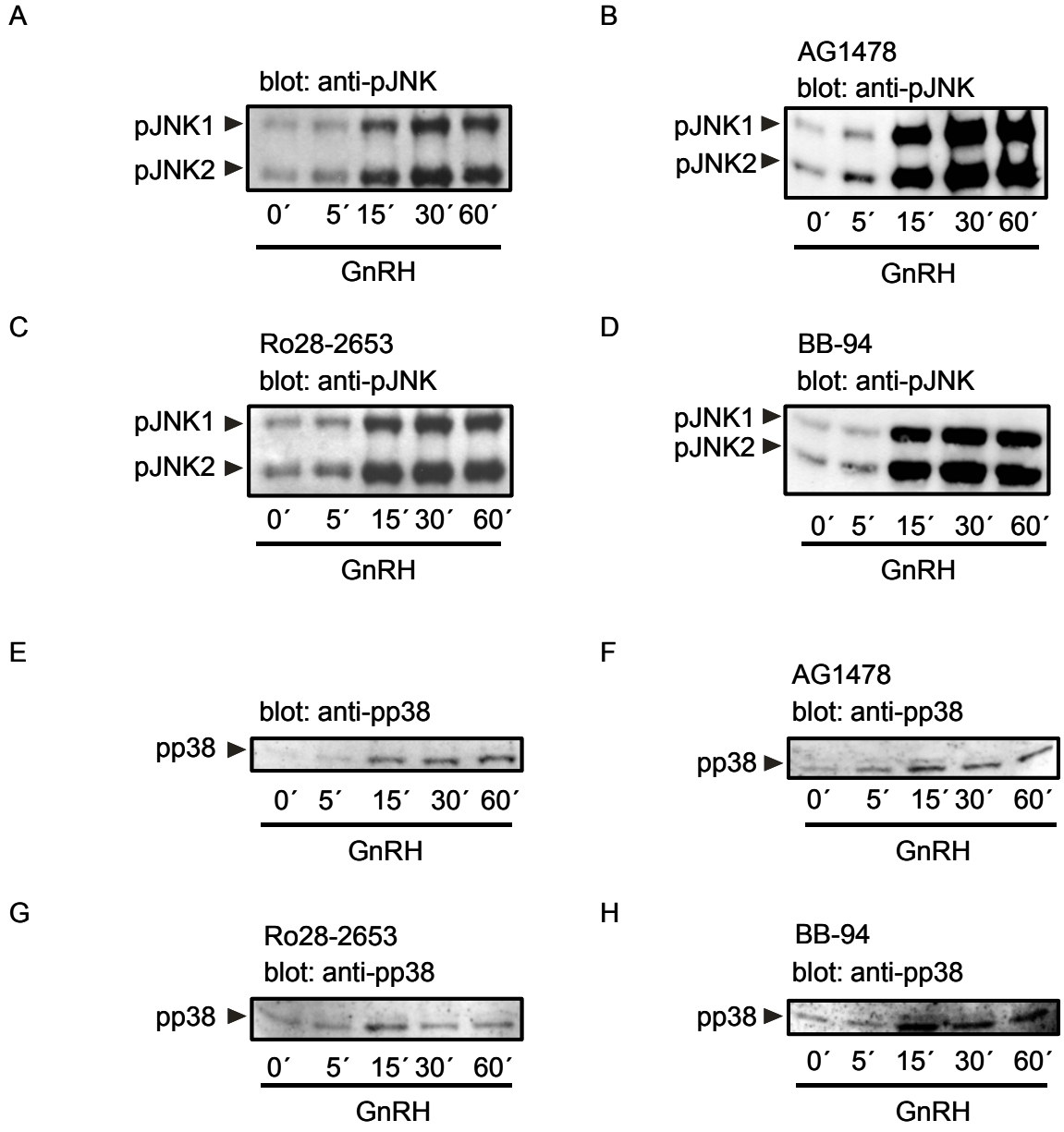


Figure 14. AG1478, Ro28-2653 or batimastat do not effect GnRH-induced JNK or p38MAPK activation in αT3-1 cells.

A: Serum-starved αT3-1 cells were stimulated with 1 µM GnRH for the indicated time periods. Western blots of cell lysates were probed with an anti-phosphoJNK antibody. **B, C, D:** Serum-starved αT3-1 cells were stimulated with 1 µM GnRH for the indicated time periods. Prior to GnRH challenge, cells were incubated with 100 nM AG1478 (B), 10 µM Ro28-2653 (C) or 10 µM batimastat (BB-94) (D) for 30 min. Western blots of cell lysates were probed with an anti-phosphoJNK antibody. **E:** Serum-starved αT3-1 cells were stimulated with 1 µM GnRH for the indicated time periods. Western blots of cell lysates were probed with an anti-phosphop38MAPK antibody. **F, G, H:** Serum-starved αT3-1 cells were stimulated with 1 µM GnRH for the indicated time periods. Prior to GnRH challenge, cells were incubated with 100 nM AG1478 (F), 10 µM Ro28-2653 (G) or 10 µM batimastat (BB-94) (H) for 30 min. Western blots of cell lysates were probed with an anti-phosphop38MAPK antibody.

In congruence with previous observations (Mulvaney 2000), an increase in the phosphorylation status of JNK1 and 2 was noted 5 min after GnRH challenge (Fig. 14 A). JNK activation reached a maximum after 30 min and remained at a comparably high level throughout the observation period. The tyrosine kinase activity of the EGFR does not appear to be required for GnRH signaling to JNK, because preincubation of cells with AG1478 had no inhibitory impact (Fig. 14 B). Along these lines, application of Ro28-2653 (Fig. 14 C) or batimastat (Fig. 14 D) left GnRH-induced JNK phosphorylation completely unaffected. According to previous studies in αT3-1 cells (Roberson 1999), GnRHR occupancy leads to p38MAPK activation commencing 15 min after agonist challenge and declining after 30 min (Fig. 14 E). Inhibition of EGFR as well as gelatinases by the specific inhibitors did not result in any decline of GnRH-mediated p38MAPK activation (Fig. 14 F, G). Furthermore, batimastat had no effect on GnRH-elicited p38MAPK activity (Fig. 14 H). In brief, gelatinase-triggered EGFR transactivation is not involved in activation of other MAPKs in αT3-1 cells. These findings further support the specificity of the inhibitors used.

4.1.8. The induction of c-fos and c-jun depends on gelatinase activity and EGF receptor transactivation.

GnRH stimulation of αT3-1 cells results in increased mRNA levels for the immediate early genes c-fos and c-jun (Cesnjaj 1994) as well as elevated c-fos and c-jun protein levels (Mulvaney 1999). The timing of c-fos and c-jun induction upon GnRH challenge, however, is discussed controversially. Hence, the time course of c-fos and c-jun induction in GnRH signaling was examined by stimulating αT3-1 cells with GnRH at multiple time points ranging from 5 min to 60 min. As depicted in Fig. 15 A,

B, a-60 min GnRH challenge resulted in a strong induction of c-fos and c-jun proteins.

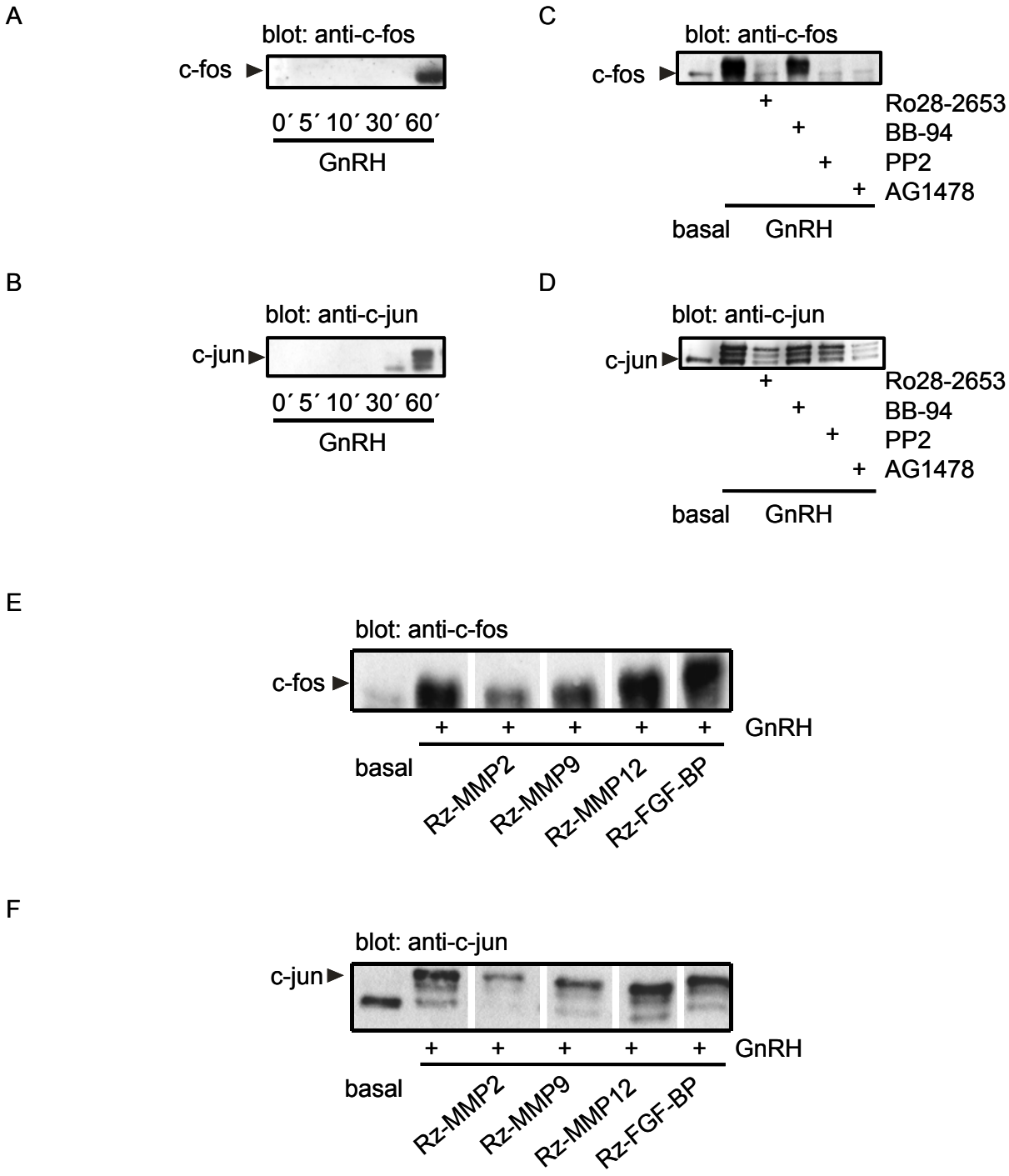


Figure 15. Induction of c-fos and c-jun in αT3-1 cells depends on gelatinase-triggered EGFR transactivation.

A, B: Serum-starved αT3-1 cells were stimulated with 1 µM GnRH for the indicated time periods. Western blots of cell lysates were probed with an anti-c-fos antibody (A) or an anti-c-jun antibody (B). **C, D:** Serum-starved αT3-1 cells were pretreated with 10 µM Ro28-2653, 10 µM batimastat (BB-94), 1 µM PP2 or 100 nM AG1478 for 30 min and subsequently treated

with 1 µM GnRH for 60 min. Western blots of cell lysates were probed with an anti-c-fos antibody (C) or an anti-c-jun antibody (D). **E, F:** αT3-1 cells were transiently transfected with ribozymes directed against MMP2 (Rz-MMP2), MMP9 (Rz-MMP9), MMP12 (Rz-MMP12) or FGF-BP (Rz-FGF-BP). Serum-depleted cells were stimulated with 1 µM GnRH for 60 min. Western blots of cell lysates were probed with an anti-c-fos antibody (E) or an anti-c-jun antibody (F).

To address the issue as to whether gelatinase activation and subsequent EGFR transactivation are of cell physiological relevance or whether the latter events are dispensable for the complex GnRH action on gonadotropic cells, c-fos and c-jun induction in the presence of various pharmacological inhibitors was examined by stimulating αT3-1 cells with GnRH for 60 min. EGFR tyrosine kinase activity was blocked by AG1478, and cells were further preincubated with the gelatinase inhibitor Ro28-2653, batimastat and the Src kinase inhibitor PP2. After GnRH challenge, Western blots were performed with antibodies recognizing c-fos and c-jun proteins (Fig. 15 C, D). Pretreatment of cells with AG1478, Ro28-2653 or PP2 profoundly counteracted the induction of c-fos and c-jun. Incubation of cells with batimastat, however, had no effect on c-jun and c-fos protein levels.

To confirm the role of MMP2 and MMP9 in GnRH-mediated c-fos and c-jun induction by an independent experimental approach, αT3-1 cells were transfected with ribozymes directed against MMP2, MMP9, MMP12 or FGF-BP. Cells treated with ribozyme Rz-MMP2 were characterized by a substantial suppression of c-fos and c-jun protein levels in response to GnRH challenge (Fig. 15 E, F). Likewise, Rz-MMP9-transfected cells displayed reduced c-fos and c-jun protein levels, although the inhibitory effect of the transfected ribozyme was less obvious when compared to Rz-MMP2-mediated gene silencing. Transfection of αT3-1 cells with ribozymes directed against MMP12 or FGF-BP did not affect GnRH-mediated gene induction.

4.2. Neuropeptides mediate growth of small cell lung cancer cells via rises in $[Ca^{2+}]_i$ and ERK activation

4.2.1. Neuropeptide-activated Pyk2 associates with Src kinases and PI3K in a $[Ca^{2+}]_i$–dependent way.

One widely accepted hypothesis is that SCLC cells originate from neoplastic transformation of neuroendocrine cells of the neural crest (Watkins 2003, Williams 1997). Many features of neuroendocrine cells, e.g. calcium-mediated synthesis and secretory release of neuropeptides and production of their cell surface receptors,

creating autocrine and paracrine growth loops (Rozengurt 1999, Williams 1997), are apparent in SCLC cells. We have previously demonstrated that galanin-induced ERK activation in SCLC cells critically depends on elevation of $[Ca^{2+}]_i$ (Wittau 2000). Therefore, we concluded that intracellular Ca^{2+}-levels besides contributing to PKC isoforms may regulate other targets to stimulate ERK activity in SCLC cells.

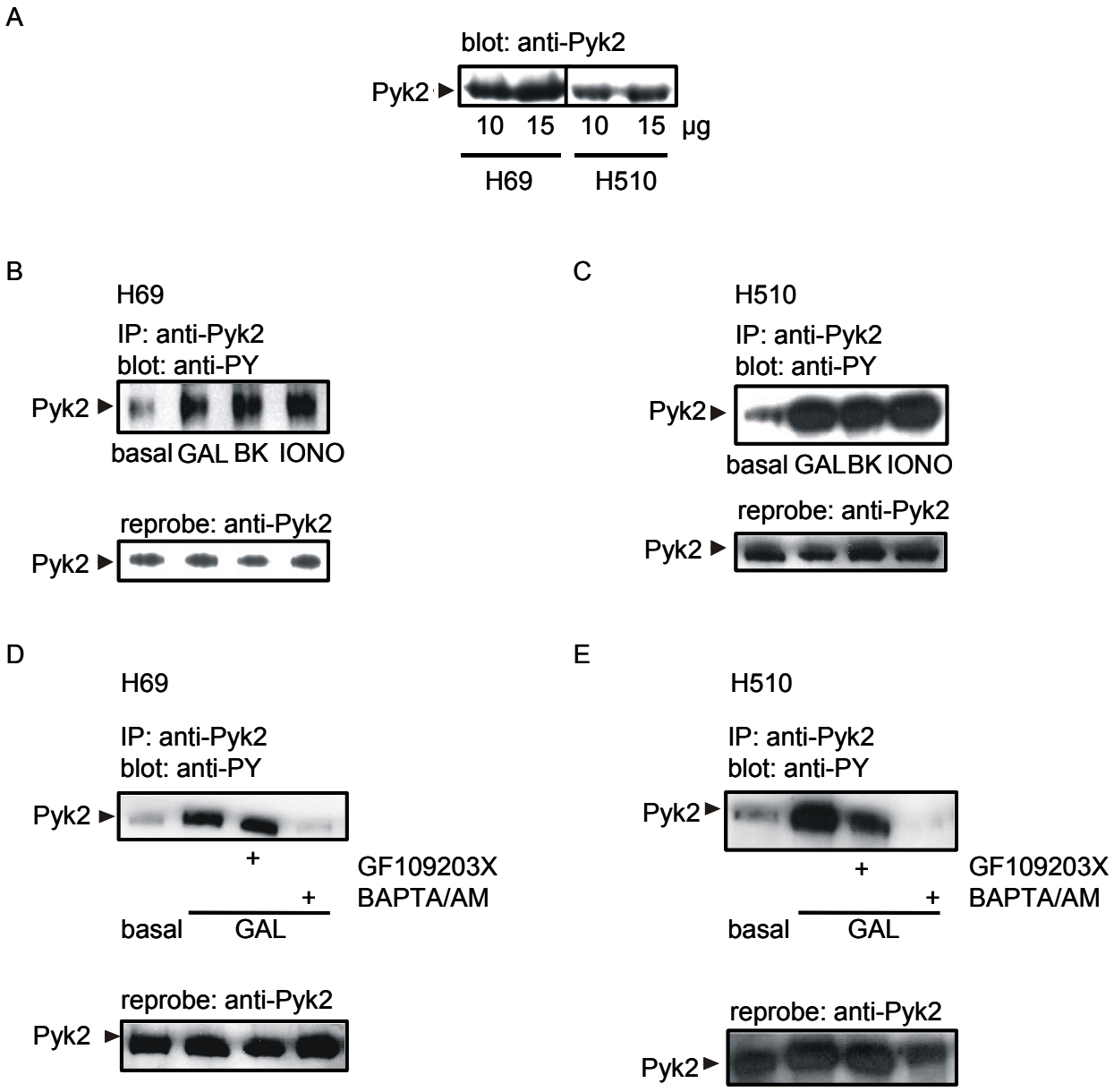


Figure 16. Neuropeptide-mediated Pyk2 activation is dependend on $[Ca^{2+}]_i$ in SCLC cells.

A: Western blots of H69 and H510 cell lysates (10 and 15 µg total protein) were probed with an anti-Pyk2 antibody. **B, C:** *Upper panels,* H69 cells (B) or H510 cells (C) were stimulated with 100 nM galanin (GAL), 1 µM bradykinin (BK) or 1 µM ionomycin (IONO) for 5 min. Pyk2 was immunoprecipitated from cell lysates with an anti-Pyk2 antibody. The immunoprecipitated proteins were then subjected to immunoblotting with an antibody against phosphotyrosine

(PY). *Lower panels*, Blots were reprobed with anti-Pyk2 antibodies. **D, E:** *Upper panels,* H69 cells (D) or H510 cells (E) were pretreated with 1 µM GF109203X or 1 µM BAPTA/AM for 30 min and subsequently stimulated with 100 nM galanin (GAL) for 5 min. Pyk2 was immunoprecipitated from cell lysates with an anti-Pyk2 antibody. The immunoprecipitated proteins were then subjected to immunoblotting with an antibody against phosphotyrosine (PY). *Lower panels*, Blots were reprobed with an anti-Pyk2 antibody.

Pyk2 is abundantly expressed in neuronal tissue and becomes activated in response to a number of extracellular stimuli including agonists acting on GPCRs thereby regulating ERK activation (Avraham 2000, Lev 1995). For this reason we tested whether Pyk2 was also expressed in SCLC cells by performing RT-PCR of H69 and H510 cell RNA. Two specific Pyk2 cDNA fragments were amplified using two different primer pairs of the Pyk2 coding region. The identity of the amplification products was confirmed by DNA sequencing (data not shown). To determine Pyk2 expression at the protein level, lysates of H69 and H510 cells containing 10 and 15 µg protein were analysed by immunoblotting with monoclonal anti-Pyk2 antibodies (Fig. 16 A). A specific band for both SCLC cell lines tested corresponding to the molecular weight of Pyk2 (116 kDa) is visible in Fig. 16 A. Next, we tested if neuropeptides like galanin or bradykinin induce Pyk2 phosphorylation in SCLC cells as assessed by increased tyrosine phosphorylation of immunoprecipitated Pyk2. Stimulation of H69 and H510 cells with either galanin (GAL) or bradykinin (BK) for 5 min leads to a rapid increase in tyrosine phosphorylation of Pyk2 (Fig. 16 B, C). This effect could be fully mimicked by treatment of cells with the calcium ionophore ionomycin (IONO) (Fig. 16 B, C). Since increases of $[Ca^{2+}]_i$ and/or active PKC are responsible for Pyk2 activation (Avraham 2000), SCLC cells were treated with the $[Ca^{2+}]_i$ chelator BAPTA/AM or the specific PKC inhibitor GF109203X prior to galanin challenge and the phosphorylation status of immunoprecipitated Pyk2 was detected with an anti-phosphotyrosine-specific antibody (Fig. 16 D, E). We observed that galanin-mediated tyrosine phosphorylation of Pyk2 was abolished after loading of H69 and H510 cells with BAPTA/AM (Fig. 16 D, E). In contrast, blockage of PKC activity by GF109203X had no inhibitory effect on galanin-induced Pyk2 tyrosine phosphorylation in H69 cells (Fig. 16 D) and showed only a slight inhibitory effect in H510 cells (Fig. 16 E). Interestingly, we coprecipitated two additional tyrosine phosphorylated bands migrating at 60 and 85 kDa which were sensitive to BAPTA/AM pretreatment as well (Fig 17 A).

It is well established that ERK activation via Pyk2 requires direct association with the SH2 domain of Src tyrosine kinases to recruit the Shc/Grb2 complex (Blaukat 1999, della Rocca 1997, Dikic 1996). Furthermore, immunoprecipitation analysis in different

cellular settings revealed complex formation between Pyk2 and PI3K (Hatch 1998, Rocic 2001). To test whether the 60-kDa fragment which was precipitated and tyrosine phosphorylated together with Pyk2 (Fig. 17 A) represents Src kinases, coprecipitation experiments were performed (Fig. 17 B). H69 cells were stimulated with galanin or treated with ionomycin for 5 min and Pyk2 was immunoprecipitated.

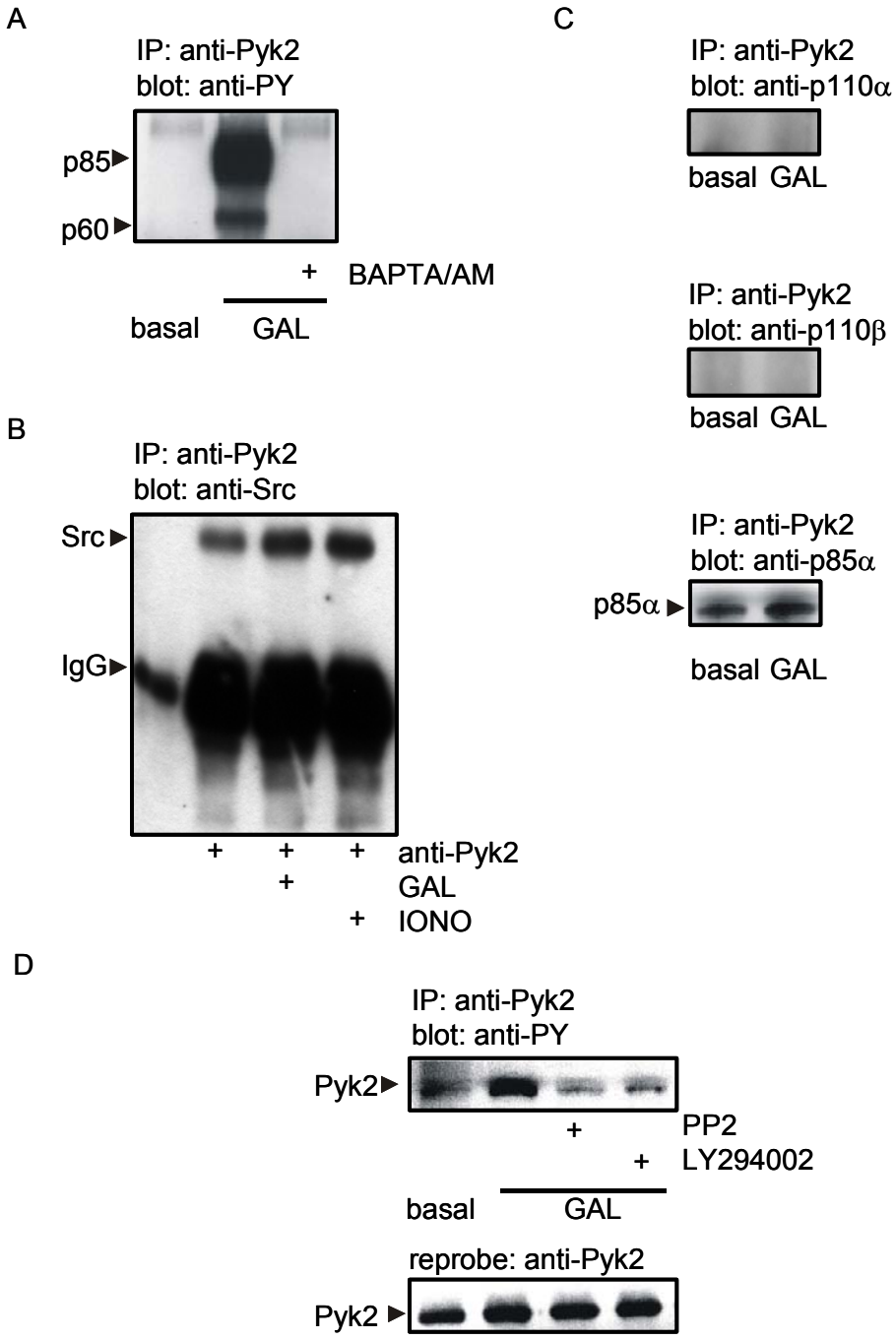


Figure 17. Pyk2 associates with Src tyrosine kinases and PI3K in a $[Ca^{2+}]_i$-dependent manner.

A: H69 cells were pretreated with 1 µM BAPTA/AM for 30 min and subsequently stimulated with 100 nM galanin (GAL). Pyk2 was immunoprecipitated from cell lysates with an anti-Pyk2 antibody. The immunoprecipitated proteins were then subjected to immunoblotting with an anti-phosphotyrosine (PY) - specific antibody. **B:** H69 cells were stimulated with 100 nM galanin (GAL) or 1 µM ionomycin (IONO) for 5 min. Cell lysates were subjected to immunoprecipitation with anti-Pyk2 antibodies, immunoprecipitates were separated on 20 cm-SDS-PAGE and detected with an antibody against Src tyrosine kinases. To demonstrate the specificity of the immunoprecipitating antibody, cell lysates were immunoprecipitated with protein A-sepharose alone (shown in first lane). **C:** H69 cells were stimulated with 100 nM galanin (GAL) for 5 min and Pyk2 was immunoprecipitated from cell lysates with an anti-Pyk2 antibody. The immunoprecipitated proteins were then subjected to immunoblotting with an antibody against PI3K p110α (upper panel), PI3K p110β (middle panel) or PI3K p85α (lower panel). **D:** *Upper panel,* H69 cells were pretreated with 1 µM PP2 or 50 µM LY294002 for 30 min and subsequently stimulated with 100 nM galanin (GAL) for 5 min. Pyk2 was immunoprecipitated from cell lysates with an anti-Pyk2 antibody. The immunoprecipitated proteins were then subjected to immunoblotting with an anti-phosphotyrosine (PY) antibody. *Lower panel*, Blots were reprobed with anti-Pyk2 antibodies.

Coprecipitated Src tyrosine kinases were subsequently detected by immunoblotting with anti-Src antibodies. Fig. 17 B shows that Src specifically associated with Pyk2 in a galanin- or ionomycin-dependent manner. Since PI3K has been shown to be important for anchorage-independent growth of SCLC cells and immunoprecipitation experiments revealed the association of PI3K p85 subunit with Pyk2 in monocytic cells (Hatch 1998, Moore 1998), we were to examine if the 85 kDa-coprecipitating band corresponds to any PI3K subunit. By using specific PI3K p110α–, PI3K p110β– or PI3K p85α–antibodies, the 85 kDa band was identified as the regulatory p85α subunit of PI3K (Fig. 17 C).

To test whether neuropeptide-triggered Pyk2 phosphorylation in SCLC cells is dependent on Src kinase- or PI3K activities, H69 cells were incubated with the specific Src kinase inhibitor PP2 or the specific PI3K inhibitor LY294002 prior to galanin stimulation (Fig. 17 D). Inhibition of Src kinase activity resulted in diminished Pyk2 tyrosine phosphorylation to basal levels (Fig. 17 D). In addition, tyrosine phosphorylation of Pyk2 was completely blocked in cells pretreated with the PI3K inhibitor (Fig. 17 D).

4.2.2. Galanin or elevation of $[Ca^{2+}]_i$ stimulate Src activity in SCLC cells.

In order to directly monitor Src kinase activity in SCLC cells, endogenous Src kinases were immunoprecipitated from H69 cell lysates and subsequently applied to an *in vitro* kinase assay using a synthesized Src-specific peptide substrate (Songyang 1995). Basal Src kinase activities in samples of H69 cells were six times higher than

in COS-7 cells whereas values for samples treated without anti-Src antibodies were below 1% of basal values. Src kinase activity could hardly be detected in samples precipitated with protein A-sepharose alone, further demonstrating the specificity of the assay. Basal values were equated to one. Stimulation of H69 cells with galanin elicited a 1.9 ± 0.4-fold increase in Src kinase activity (Fig. 18 A) which was inhibited to basal levels by depletion of intracellular calcium with BAPTA/AM (Fig. 18 A). Treatment of cells with ionomycin alone was sufficient to activate Src kinases (Fig. 18 A). $[Ca^{2+}]_i$-dependent activation of Src kinase activity was abolished by pretreatment of cells with the Src kinase inhibitor PP2 (Fig. 18 A). Similar results were obtained by stimulating Src kinase activity in H510 cells (Fig. 18 B). Treatment of H510 cells with galanin or ionomycin led to increased Src kinase activities which were sensitive to BAPTA/AM- or PP2 preincubation, respectively (Fig. 18 B).

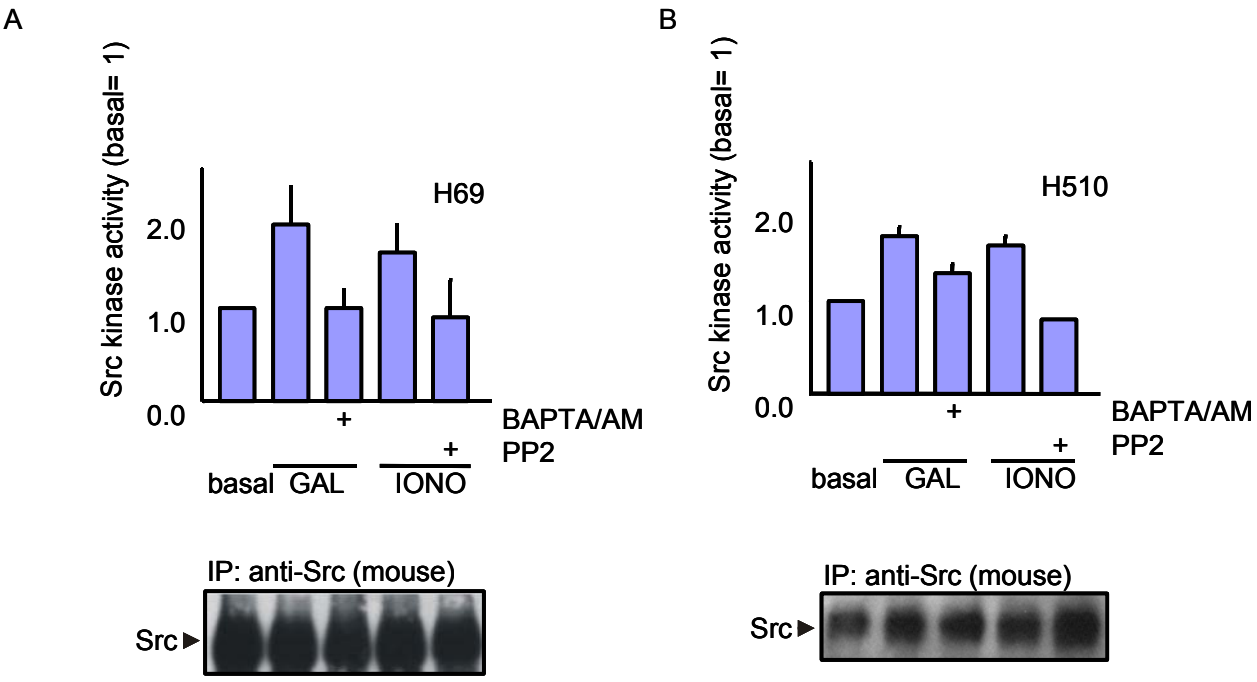


Figure 18. Galanin or elevation of $[Ca^{2+}]_i$ stimulate Src activity in SCLC cells.

A, B: *Upper panels*, H69 (A) or H510 (B) cells were stimulated with 100 nM galanin (GAL) or 1 µM ionomycin (IONO) for 5 min in the presence or absence of 1 µM BAPTA/AM or 1 µM PP2, respectively. Src kinases were immunoprecipitated with an anti-src antibody and src kinase activity was measured in an *in vitro* kinase assay. The data are presented as ± SEM of at least three independent experiments performed in duplicates and are expressed as –fold stimulation relative to mean basal Src kinase activity. *Lower panels*, Aliquots of lysates used for Src kinase activity assays were subjected to immunoprecipitation with an anti-Src kinase antibody and blotted with an anti-Src kinase antibody. One representative blot is shown.

4.2.3. Neuropeptide-induced Ras activation is dependent on $[Ca^{2+}]_i$ and Src kinase activity with RasGEFs not being involved.

As explained before, ERK activation by agonists acting on $G_{q/11}$-coupled receptors can be accomplished via Ras-independent and –dependent pathways by direct phosphorylation of Raf-1 by PKC isoforms or GTP-loading of Ras, respectively (Gudermann 2000, Kolch 1993). A role for $[Ca^{2+}]_i$ in the activation of Ras has been reported to proceed indirectly through activation of Pyk2 or Ca^{2+}-dependent RasGEFs, RasGRF or RasGRP (Murasawi 1998, Zwartkruis 1999). RasGEFs are predominantly expressed in neuronal tissue with RasGRF2 also being highly expressed in lung tissue (Cullen 2002, Table 1). In order to decipher the expression of RasGEFs in SCLC cells, total RNA from H69 and H510 cells was isolated and RT-PCR was performed using specific primer pairs for RasGRF2 and RasGRP/CalDAG-GEF II (Fig. 19 A). Amplification products of the correct size were obtained only with oligonucleotides specific for RasGRF2 (Fig. 19 A). The identity of the PCR products was confirmed by DNA sequencing. Amplification of the housekeeping gene GAPDH was positive (Fig. 19 A).

Activating *ras* mutations have not been described in SCLC (Mitsudomi 1991, Suzuki 1990) and overexpression of inducable Raf-1 activity or constitutively active Ras in SCLC cells rather causes growth arrest or increased levels of endocrine differentiation markers, respectively (Mabry 1988, Ravi 1998). These data suggest that Ras activation may not award a growth advantage to SCLC cells. Furthermore, oncogenic Ras strongly activates JNK in SCLC cells whereas stimulation of ERK activity was only modestly affected (Xiao 2000). Given that the effect of neuropeptide stimulation on GTP-loading of Ras has not been investigated in SCLC cells, H69 cells were stimulated with galanin or bradykinin for 5 min and the GTP-loaded form of Ras was precipitated by using a GST-fusion protein containing the minimal Ras-binding domain of Raf-1 (Fig. 19 B, C). As shown in Fig. 19 B, C, both neuropeptides induced a rapid activation of Ras which was dependent on $[Ca^{2+}]_i$ and Src kinase activity as assessed by pretreatment of cells with BAPTA/AM or PP2, respectively. To examine the involvement of RasGRF2 in Ca^{2+}-dependent signaling to Ras, cells were incubated with the calmodulin antagonist calmidazolium chloride prior to galanin or bradykinin challenge (Fig. 19 B, C). Inhibition of calmodulin did not decrease neuropeptide-induced GTP-loading of Ras (Fig. 19 B, C).

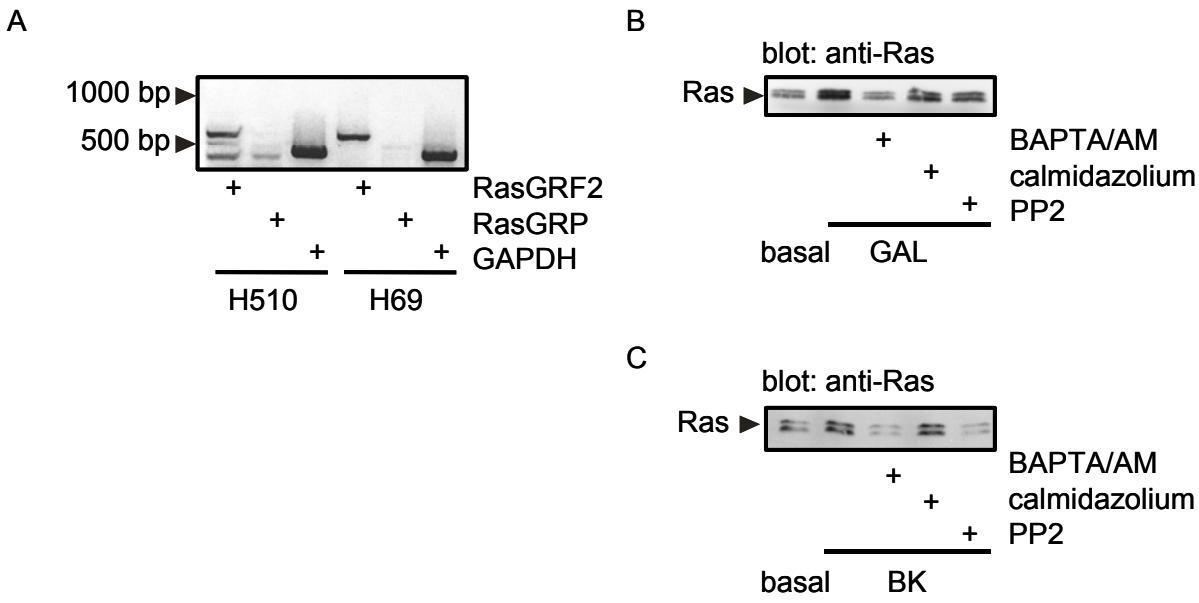


Figure 19. Ras activity is induced by neuropeptides in a $[Ca^{2+}]_i$-dependend manner with Ras exchange factors not being involved in SCLC cells.

A: Total RNA of H69 and H510 cells was isolated and RasGRF2, RasGRP and GAPDH cDNAs were amplified by RT-PCR. The DNA size is indicated at the left. **B, C:** H69 cells were pretreated with 1 µM BAPTA/AM, 1 µM calmidazolium chloride or 1 µM PP2 for 30 min and subsequently stimulated with 100 nM galanin (GAL; A) or 1 µM bradykinin (BK; B) for 5 min. Cell lysates were incubated with GST-RBD precoupled to glutathione-sepharose at 4°C for 30 min. GTP-loaded Ras was analysed by SDS-PAGE followed by immunoblotting with an anti-Ras antibody.

4.2.4. Src kinase activity is required for neuropeptide-induced ERK activation and anchorage-independent growth of SCLC cells.

Challenging H69 cells with galanin for 5 min leads to an increase in ERK activity (Wittau 2000). Since neuropeptide-mediated stimulation of ERK activity represents an essential mechanism for the growth of SCLC cells (Rozengurt 1999), we next addressed the role of Src kinase activity in mediating ERK activation in SCLC cells. Endogenous ERK activity of H69 cells was measured in an *in vitro* kinase assay using myelin basic protein as a substrate (Grosse 2000b). Experimental values of non-stimulated H69 cells were equated to one. Stimulation of H69 cells with galanin induced a 2.1 ± 0.3-fold increase of ERK activity (Fig. 20). Moderate stimulation of ERK activity was obtained by bradykinin treatment (Fig. 20). Neuropeptide-induced ERK activities were blocked by preincubating the cells with PP2 (Fig. 20).

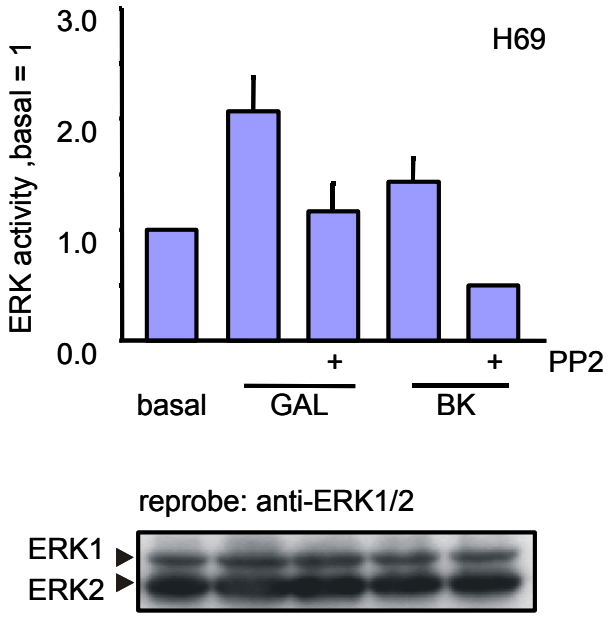


Figure 20. ERK activity of H69 cells is dependent on Src kinase activity.

Upper panel, H69 were pretreated with 1 µM PP2 for 30 min and subsequently treated with 100 nM galanin (GAL) or 1 µM bradykinin (BK) for 5 min. ERK1/2 were immunoprecipitated with an anti-ERK1/2 antibody and ERK activity was measured in an *in vitro* kinase assay. The data are presented as ± SEM of three independent experiments performed in duplicates and are expressed as –fold stimulation relative to mean basal ERK kinase activity. *Lower panel*, Aliquots of lysates used for ERK activity assays were used for immunoblotting with an anti-ERK1/2 antibody. One representative blot is shown.

A well characterized marker for the transforming potential of cells is represented by the ability to grow anchorage-independently (Sethi 1991). Anchorage-independent growth can be analysed by means of colony formation in semisolid medium (soft agar). The invasiveness of the tumor in specimens taken from SCLC strongly correlates with generation of tumor cell colonies in agarose (Carney 1980). To elucidate the biological consequences of Src kinase inhibition on SCLC growth in soft agar, we examined the effect of PP2 on clonogenic growth of H69 and H510 cells. Initially, concentration-response experiments were performed. H69 cells were seeded in soft agar supplemented with galanin in the presence of PP2 in concentrations ranging from 10^{-3} – 1 µM. Colonies from at least three independent experiments including ten separate dishes were counted after 21 days. One representative experiment is shown in Fig. 21 A. Galanin stimulated enhanced clonogenic growth of H69 cells in soft agar (21 A). PP2 treatment resulted in a clear concentration-dependent decrease in galanin-induced colony formation of H69 cells (IC_{50} = 10^{-2} µM, Fig. 21 A). As demonstrated in Fig. 21 B- E, PP2 inhibited basal growth of H69 and

H510 cells. The addition of galanin or bradykinin failed to overcome PP2-induced growth inhibition with the inhibitory effect being less pronounced in clonogenic assays with H510 cells whereas cells not treated with PP2 efficiently responded to neuropeptide-mediated growth (Fig. 21 B - E).

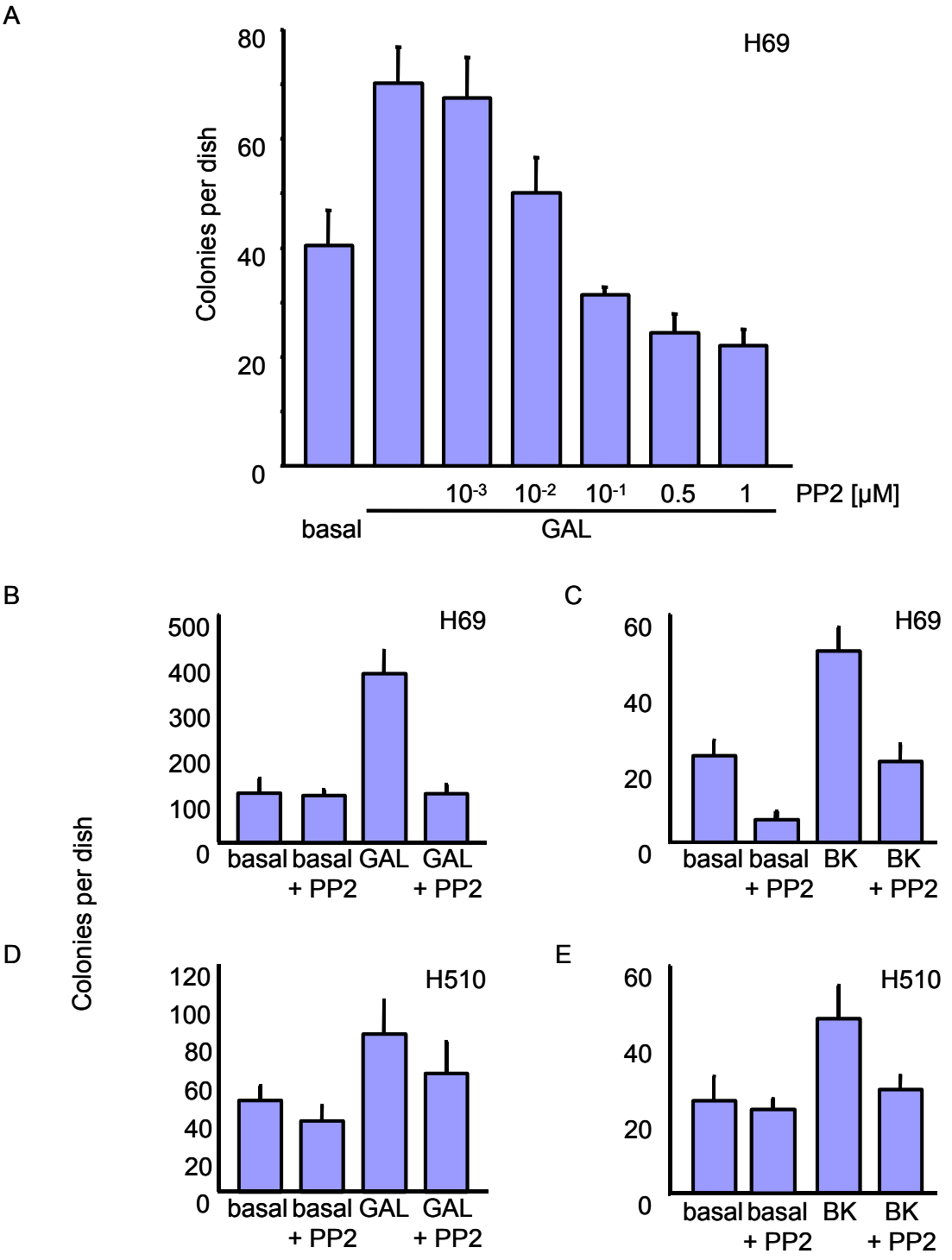


Figure 21. Colony formation of SCLC cells is dependent on Src kinase activity.

A: A single-cell suspension of H69 cells was plated in HITESA medium containing agarose at a density of $1x10^4$ cells/dish in the presence or absence of 10^{-3} – 1 µM galanin and was incubated for 21 days. **B, C:** A single-cell suspension of H69 cells was plated in HITESA medium containing agarose at a density of $1x10^4$ cells/dish in the presence or absence of 100 nM galanin (GAL, B) or bradykinin (BK, C) and/or 1 µM PP2 and incubated for 21 days. **D, E:** A single-cell suspension of H510 cells was plated in HITESA medium containing agarose at a density of $1x10^4$ cells/dish in the presence or absence of 100 nM galanin (GAL, D) or 1 µM bradykinin (BK, E) and/or 1 µM PP2 and incubated for 21 days. *Columns,* mean number of colonies formed on ten separate dishes, *bars*, SEM. In all cases, one reprensentative out of three independent experiments is shown.

To further describe the inhibitory effect of PP2 under biological conditions, liquid culture assays of SCLC cells were performed (Fig. 22). Cells were incubated in culture medium and cell numbers were calculated after 7 days. The addition of PP2 to H69 and H510 cells in liquid culture resulted in decreased cell numbers below basal levels (Fig. 22). Galanin or bradykinin added to the culture media did not result in increased cell numbers, in contrary to cell counts elicited by galanin or bradykinin alone (Fig. 22).

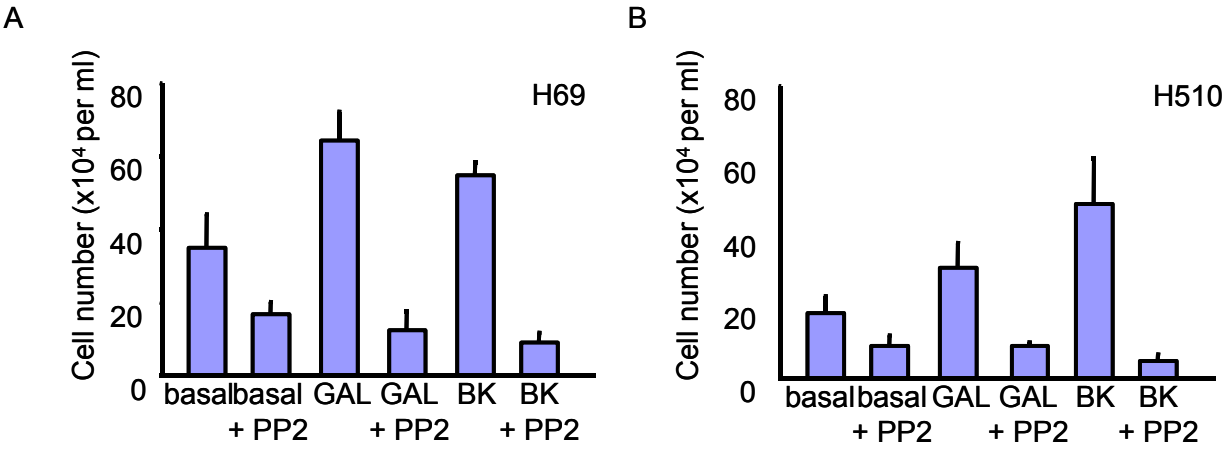


Figure 22. Growth of SCLC cells in liquid culture is dependent on Src kinase activity.

A, B: H69 (A) and H510 (B) cells were aliquoted in 24-well plates in HITESA medium in the presence or absence of 100 nM galanin (GAL) or 1 µM bradykinin (BK) and/or 1 µM PP2. Cell numbers were counted from a minimum of 3 wells per condition at day 7. *Columns,* mean number of cells, *bars*, SEM. In all cases, a reprensentative out of three independent experiments is shown.

4.2.5. Overexpression of Pyk2 induces cell death in SCLC cells.

To gain further insight into the signaling properties of Pyk2 in SCLC cells, we were to overexpress Pyk2 and various Pyk2 mutants in SCLC cells. The presence of potential SH2 and SH3 binding motifs as well as proline-rich regions mediates the

association of Pyk2 with a number of proteins (Schlaepfer 1999). Tyrosine 402 of Pyk2 represents the major autophosphorylation and binding site for the Src SH2 domain (Schlaepfer 1999). Pyk2 mutants deficient in this residue have been shown to lack their ability to autophosphorylate as well as form complexes with Src kinases and cells overexpressing Pyk2-Y402F are impaired in stimulation of ERK activity (Blaukat 1999). Kinase-dead mutant Pyk2-K457A(PKM) has been shown to be unable of activating Src (Blaukat 1999). In cells transfected with Pyk2-PKM no Grb2/Sos association and ERK activation was detectable (Blaukat 1999, Lev 1995). Since the above mentioned domains link Pyk2 to the ERK cascade, we overexpressed Pyk2-wt, Pyk2-Y402F and Pyk2-PKM in SCLC cells. In initial experiments Pyk2-wt was subcloned into the retroviral expression vector pLXSN and recombinant retroviruses were produced. Unexpectedly, infection of SCLC cells with these viruses resulted in spontaneous cell death. On account of this, we chose a transient transfection approach. Because the transfection efficiency was below 10%, we were not able to perform biochemical assays with transfected SCLC cells. Therefore, the morphology of single cells was analysed by fluorescent microscopy using bisbenzimide as a fluorescent dye that binds to DNA in cell nuclei. Cells harboring characteristic features of apoptosis such as membrane blebbing, cell swelling or DNA degradation visible as apoptotic bodies were designated apoptotic. Initially time-course experiments were conducted. H69 cells were transfected with Pyk2-wt and cells were monitored 12 h, 24 h, 36 h and 48 h after transfection. An increased rate of apoptotic cells was first evident at about 36 h after transfection. Pyk2-wt transfected H69 cells showed characteristic morphological features of apoptosis (Fig. 23 A). Surprisingly, after 36 h 72.6% ± 18.2% of all cells expressing Pyk2-wt underwent apoptosis in contrast to 3.2% ± 6.7% mock (pcDNA3.1) transfected cells (Fig. 23 A, B). 65.8% ± 21.6% or 61.8% ± 21.4% cells expressing PYK2-PKM or PYK2-Y402F, respectively, were apoptotic (Fig. 23 A, B). In control experiments with PC12W cells, overexpression of Pyk2-wt (98.7% ± 5.2% viable cells), Pyk2-PKM (98.2% ± 4.5% viable cells) or Pyk2-Y402F (99.1% ± 2% viable cells) showed no induction of apoptosis (mock transfected cells 99.4% ± 2.1% viable cells, Fig. 23 C). Since Pyk2 mutated at tyrosine residue 881 has been shown to fully stimulate Src and ERK activities (Blaukat 1999), we did not include this Pyk2 mutant in our studies.

In SCLC cells, besides coupling to G proteins of the $G_{q/11}$ family, neuropeptide receptors initiate the Rho pathway by coupling to $G_{12/13}$ proteins (Wittau 2000). Graf and PSGAP are GAPs for RhoA and Cdc42 functioning as mediators for Pyk2 to regulate Rho-family GTPases (Hildebrand 1996, Ren 2001). Both GAPs associate

with Pyk2 by binding to the second proline-rich domain (Ohba 1998, Ren 2001). To examine the possible involvement of the Rho pathway in initiating an apoptotic cell response after overexpression of Pyk2, H69 cells were transfected with Pyk2-P859A (Ohba 1998). 58.9% ± 13.4% of H69 cells overexpressing Pyk2-P859A underwent apoptosis (Fig. 23 A, B) whereas no apoptosis was detectable in PC12W cells (100.0% ± 0.0% viable cells, Fig. 23 C).

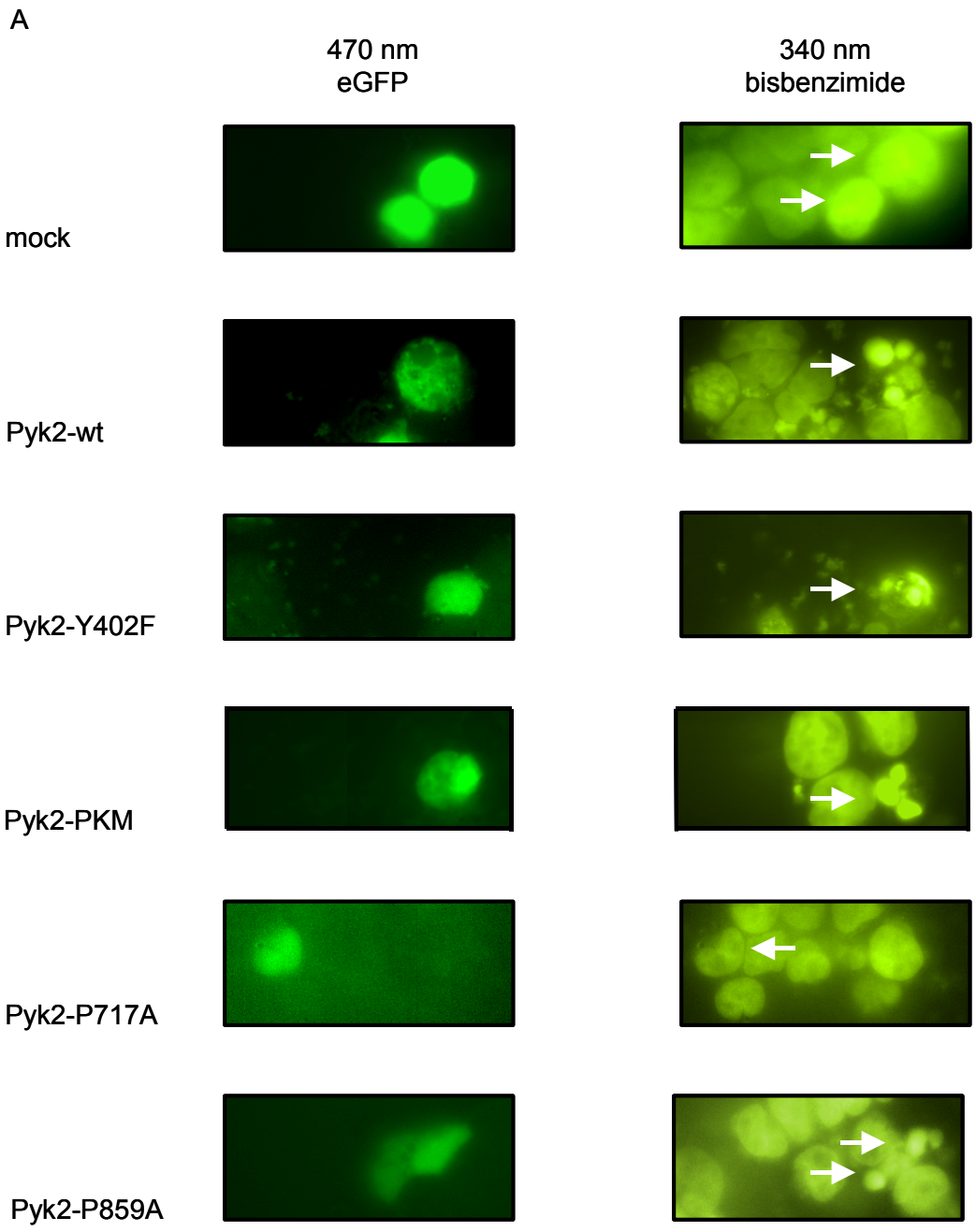


Figure 23. Overexpression of Pyk2 leads to apoptosis in H69 cells.

H69 cells were transfected with plasmids encoding Pyk2-wt, Pyk2-Y402F, Pyk2-PKM, Pyk2-P717A and Pyk2-P859A. Mock (pcDNA3) transfected cells served as controls. To allow detection of transfected cells, eGFP was cotransfected as a marker. **A:** After fixation, transfected cells were identified at 470 nm and cell morphology was examined at 340 nm using a fluorescence miscroscopy device.

It has been reported that Pyk2 is involved in activation of the JNK pathway and the $p130^{Cas}$/Crk complex specifically links Pyk2 to it (Astier 1997, Blaukat 1999, Dolfi 1998, Yu 1996). Proline residue 717 located in the first proline-rich domain of Pyk2 has been shown to mediate the association of Pyk2 with $p130^{Cas}$ (Ohba 1998). To determine whether the JNK pathway is involved in cell death observed after transfection of Pyk2-wt, Pyk2 mutated at proline 717, Pyk2-P717A (Ohba 1998), was transiently transfected in H69 cells (Fig. 23 A, B). Pyk2-P717A overexpression did not induce cell death in H69 cells (12.4% ± 11.7% apoptotic cells, Fig. 23 A, B). In control experiments with PC12W cells transfection of Pyk2-P717A showed no loss of cell viability (97.5% ± 4.5% viable cells, Fig. 23 C).

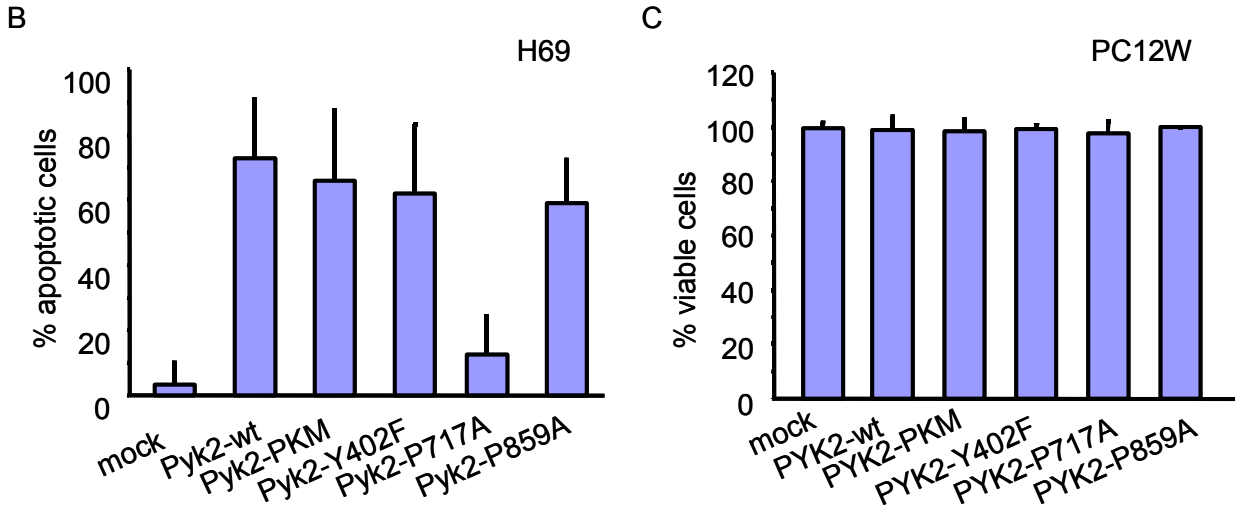


Figure 23. B: The percentage of apoptotic H69 cells (mean ± SEM) among positively transfected cells from at least three independent experiments are shown. **C:** The percentage of viable PC12W cells (mean ± SEM) among positively transfected cells from at least three independent experiments are shown.

So far, JNK activation by neuropeptides has not been described for SCLC cells because JNK activation rather occurs successively to ultraviolet radiation in these cells (Butterfield 1997). Since our data indicate an involvement of the JNK pathway in Pyk2-induced cell death, H69 cells were treated with galanin ranging from 5 to 60 min and phosphorylation of JNKs was determined in Western blot experiments with an anti-phosphoJNK-specific antibody. As shown in Fig. 23 D, H69 cells do not

exhibit any basal JNK activity. JNK phosphorylation started 5 min after galanin treatment and declined within 15 min reaching basal levels 60 min after galanin stimulation (Fig. 23 D). On the contrary, H69 cells display a high basal phosphorylation status of ERKs as assessed by Western blotting with an anti-phosphoERK antibody (Fig. 23 E).

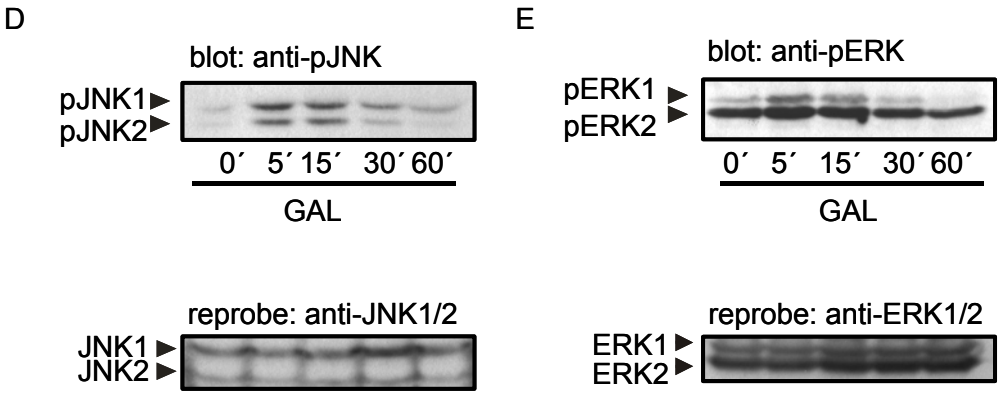


Figure 23. D, E: *Upper panels*, H69 cells were stimulated with 100 nM galanin (GAL) for the indicated time periods. Western blots of cell lysates were probed with an anti-phosphoJNK (D) or anti-phosphoERK (E) antibody. *Lower panels*, Blots were reprobed with anti-JNK1/2 (D) or anti-ERK1/2 (E) antibodies.

5. DISCUSSION

By binding to their cognate GPCRs, many potent mitogens such as neuropeptides, angiotensin II or lysophosphatidic acid stimulate cell proliferation via engaging the ERK/MAPK cascade. As mentioned before, agonists stimulating $G_{q/11}$-coupled receptors activate PLCβ isoforms thereby activating PKCs and elevating $[Ca^{2+}]_i$. These two second messengers represent key molecules for coupling $G_{q/11}$ proteins to the ERK/MAPK cascade. In this work, by means of GnRH in gonadotropic αT3-1 cells and galanin or bradykinin in SCLC cells, different aspects of $G_{q/11}$-dependent mitogenic signaling pathways were revealed. Our findings together with previous reports underline the notion that signaling pathways emanating from $G_{q/11}$-coupled receptors are tightly regulated in a cell- and receptor-specific manner.

5.1. Matrix metalloproteinases 2 and 9 mediate EGF receptor transactivation by gonadotropin-releasing hormone

Previously, we have shown that GnRH-elicited EGFR transactivation in αT3-1 cells is required for Ras activation and accelerates the kinetics of GnRH-induced ERK activity (Grosse 2000b). In the present study, we extend our earlier observations and delineate the signal transduction pathway connecting the agonist-occupied GnRHR with the EGFR (Fig. 24). Recent work on the cross-talk between EGFR and GPCRs has suggested a pivotal contribution of MMPs to this kind of receptor cross-communication. Using non-selective MMP inhibitors, it was shown that GPCR stimulation resulted in cleavage of EGFR ligand-precursors and production of diffusible growth factors which then activate the EGFR (Carpenter 2000a). LPA- and carbachol-induced EGFR activation in COS-7 cells as well as bombesin-mediated EGFR phosphorylation in PC-3 cells was found to be batimastat-sensitive (Prenzel 1999). Prostaglandin E_2-mediated activation of EGFR in gastric epithelial- and colon cancer cells was inhibited by another broad-spectrum metalloproteinase inhibitor, GM6001 (Pai 2002). While the principal involvement of MMPs in the EGFR transactivation process appears to be a rather undisputed issue, the identity of the proteolytic enzymes and their regulation via GPCR ligands still remains elusive. Recently, three members of the ADAM family of proteases, i. e. ADAM 10, ADAM12 and ADAM17/TACE, have been claimed responsible for shedding of EGF-like ligands resulting in EGFR transactivation (Asakura 2002, Gschwind 2003, Lemjabbar 2002). While MMP3 has the potential to cleave HB-EGF precursors to release active growth

factors (Suzuki 1997), it has not been possible to conclusively ascribe a defined role in EGFR/GPCR cross-talk to distinct members of the MMP family.

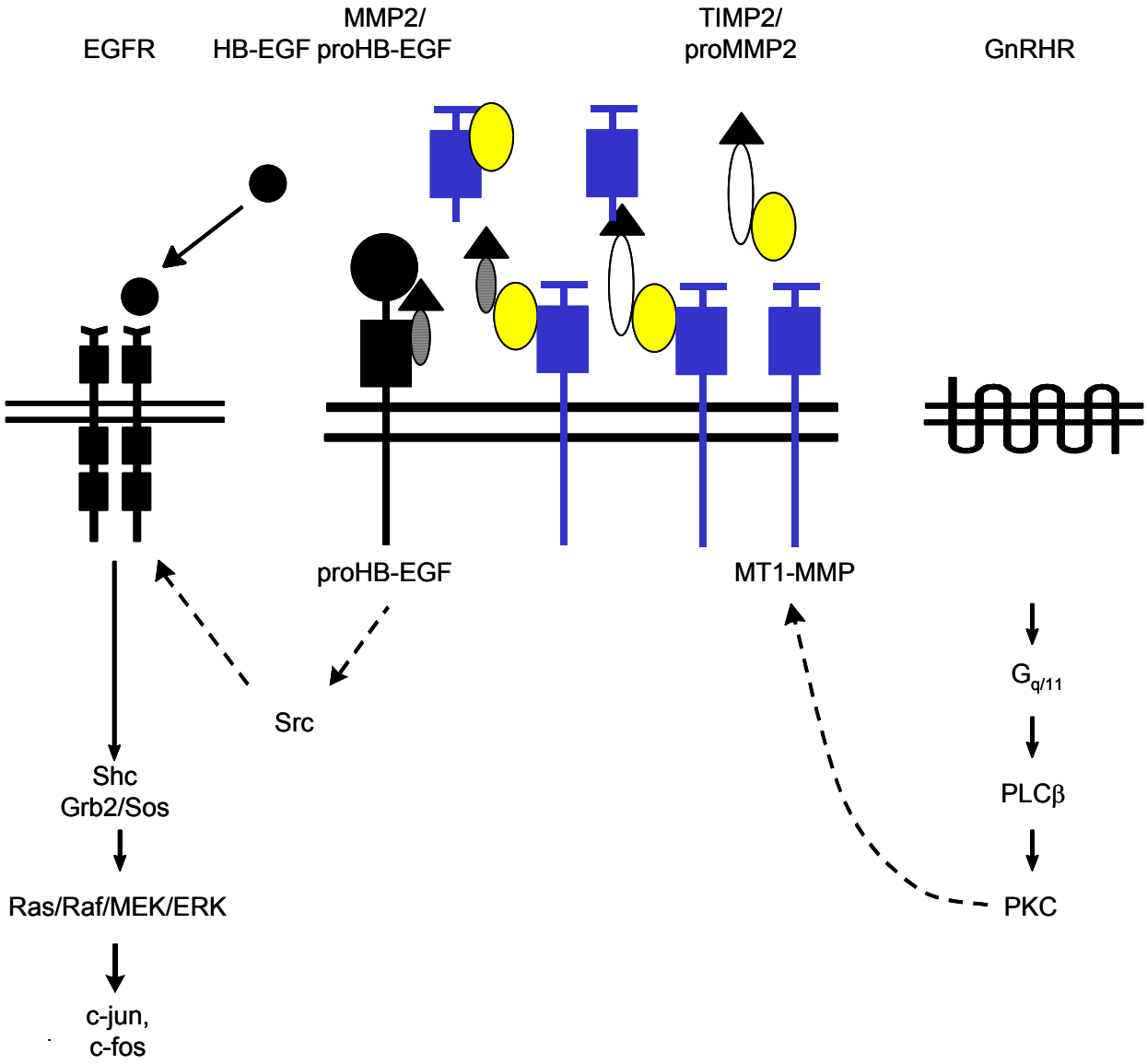


Figure 24. Cross-communication between GnRHR and EGFR in gonadotropic cells. GnRHR-induced gelatinase and MT1-MMP activation leads to shedding of proHB-EGF resulting in EGFR transactivation. GnRHR/EGFR cross-communication engages the Ras/MEK/ERK cascade via Src kinases. It might be speculated that the agonist-occupied GnRHR regulates MT1-MMP function by a mechanism utilizing PKC as indicated by the dashed line.

The expression of gelatinases MMP2 and MMP9 can be demonstrated in a variety of human tumors and a major impact of these enzymes on the acquisition of a malignant phenotype during multiple stages of tumor progression has been emphasized (McCawley 2000). For instance, upregulation of MMP2 and MMP9 has

been correlated with increased invasiveness of different tumors including pituitary adenomas and carcinomas (Paez-Pereda 2000). Elevated levels of gelatinases as well as of the membrane-spanning MT1-MMP and the endogenous MMP inhibitors TIMP1 and TIMP2 were noted in pituitary adenomas (Paez-Pereda 2000). Furthermore, MMP9 expression in pituitary tumors was related to aggressive biological behavior and the incidence of MMP9 secretion was found to be significantly higher in invasive pituitary adenomas and carcinomas as compared to non-invasive tumor species (Kawamoto 1996, Turner 2000). In light of these observations, we first concentrated on a specific gelatinase inhibitor, Ro28-2653, to dissect the signaling pathway linking the GnRHR to the ERK family of MAPKs in gonadotropic cells. Inhibition of MMP2 and MMP9 activities with Ro28-2653 profoundly suppressed GnRH-induced mitogenic signaling events in αT3-1 cells including EGFR phosphorylation, phosphorylation of Src kinases, GTP-loading of Ras, stimulation of ERK activity and induction of transcription factors like c-fos and c-jun.

To unequivocally identify MMP2 and MMP9 as the relevant proteolytic enzymes involved in the cross-communication between GnRHR and EGFR in αT3-1 cells, we opted for a novel ribozyme approach to specifically ablate MMP2 and MMP9 expression. A low molecular weight polyethylenimine (PEI) was used as a transfection reagent to stabilize and introduce unmodified bioactive all-RNA ribozymes into cells (Aigner 2002). In contrast to high molecular weight PEIs, low molecular PEIs display a far less cytotoxicity in combination with higher transfection efficiencies. PEIs are able to condense and compact ribozymal RNA into complexes which are then transported into the cell through endocytosis. We have recently shown in our laboratory that PEI-complexed ribozymes are bioactive intracellularly as demonstrated by efficient down-regulation of mRNA and protein levels of various gene products *in vitro* and *in vivo* (Aigner 2002). GnRH-induced EGFR phosphorylation was completely prevented in cells transiently transfected with ribozymes directed against MMP2. Rz-MMP9-loaded cells showed a substantial reduction of EGFR phosphorylation. Specificity of the experimental approach was demonstrated by using ribozymes directed against signaling components presumably unrelated to the signaling cascade under study, namely MMP12 and FGF-BP. The latter measure did not entail any inhibitory effect on GnRH-induced EGFR activation. Thus, the results obtained by ribozyme transfection were in perfect agreement with the conclusions drawn from pharmacological inhibition of MMPs. In contrast to our findings, the blockage of EGFR tyrosine kinase activity by the selective tyrphostin

AG1478 has been reported not to affect ERK activation via the GnRHR in the same cell line calling into question the relevance of EGFR transactivation for GnRH signalling to ERKs (Benard 2001). So far, the discrepancy between the two studies remains unresolved. Of note, GnRH-induced signaling to ERKs in hypothalamic neurons critically depends on EGFR transactivation (Shah 2003a).

The exact mechanism and regulation of growth factor release resulting in EGFR transactivation is not fully understood. EGFR ligands are initially synthesized as membrane-bound precursor proteins which are subsequently processed by metalloproteinase activities yielding a secreted product. Conversion of insoluble precursors to soluble factors by enzymatic cleavage may constitute an important post-translational modification regulating growth factor activity and availability (Goishi 1995, Sunnarborg 2002). In CHO cells, for instance, shedding of membrane-anchored HB-EGF can effectively be induced by phorbolesters and subsequent PKC activation. However, the shedding process appears to be regulated by ERK, and time course experiments indicated that TPA/PKC-induced ERK activation precedes HB-EGF release (Gechtman 1999). In gonadotropic cells examined by us, however, MT1-MMP secretion, gelatinase activation and HB-EGF shedding is a rapid process whose time course concurred with that of GnRH-dependent Ras and ERK activation. Here, we directly show for the first time MT1-MMP secretion and gelatinase activation as an immediate response to occupation of a $G_{q/11}$-coupled receptor by its agonist. Protein levels of active gelatinases in conditioned media were found to be elevated within 5 min of GnRH stimulation together with migration of MT1-MMP from cells to the cell culture media. Furthermore, gelatin zymography carried out with conditioned media of αT3-1 cells demonstrated increased MMP2 enzyme activity following short-term stimulation of cells with GnRH.

At present, the activation mechanism of gelatinases in gonadotropic cells still remains elusive. MMP2 belongs to the most abundant MMP family and is constitutively secreted in a latent form by many cell types. MMP2 is set apart from other MMPs in that its proform is not activated by enzymatic digestion with exogenous proteinases, but requires the activity of MT-MMPs. The activation of proMMP2 is potentiated by the formation of a trimolecular complex, consisting of MT1-MMP, TIMP2 and proMMP2 thought to cause a sufficient local concentration of proMMP2 allowing activation by TIMP2-free MT1-MMP and ensuing autocatalytic processing to the fully active form (Butler 1998, English 2001). Data presented here show that downregulation of MT1-MMP or MMP2 results in diminished MMP2 or MMP9 activation, respectively. MT1-MMP has been identified as an ectoenzyme generated by cleavage at the cell surface. Data strongly support the existence of at

least three forms of MT1-MMP: membrane-associated, furin-processed and soluble. The latent soluble form of MT1-MMP has been ascribed fully active. Rapid elevation of MT1-MMP in culture media after GnRH challenge can be explained by MT1-MMP being processed at the cell surface, then complexed to TIMP2 and the complex released in the conditioned medium after proMMP2 activation (Kazes 1998, Sato 1996, Toth 2002, Will 1996). MMP2 released from the cell surface is then assumed to convert proMMP9 into its active form (Westermarck 1999).

There is considerable evidence supporting a role of Src family tyrosine kinases in the signaling pathways from GPCRs to RTKs (Grosse 2000b). In GnRHR-expressing GT1-7 neurons, it has recently been suggested that Src may act upstream of the transactivated EGFR (Shah 2003b). However, the contribution of the latter tyrosine kinase to EGFR activation by GPCRs is a highly contentious issue. In the present study, we show that GnRH-induced EGFR transactivation is clearly affected by PP2-mediated Src blockage. Inhibition of gelatinase activities either by pharmacological or ribozymal strategies diminished phosphorylation of Src kinases after GnRH challenge whereas inhibition of EGFR kinase activity had no impact on the phosphorylation status of Src. These findings demonstrate that Src is involved in GnRH-mediated EGFR tyrosine phosphorylation and has to be placed downstream of gelatinases and upstream of the EGFR in the GnRH signaling cascade in αT3-1 cells.

We have previously pointed out that GnRHR/$G_{q/11}$-induced ERK activation in αT3-1 cells proceeds via activation of Ras GTPases. A dominant-negative Ras mutant overexpressed in COS-7 cells, interfered $G_{q/11}$-mediated ERK activation. Stimulation of Ras was dependend on PKC activity. By applying PP2 to αT3-1 cells, we were able to show the participation of Src kinases in GnRH-induced GTP-loading of Ras. This was additionally underlined by overexpression of Csk in COS-7 cells transiently transfected with the GnRHR resulting in diminished GTP-loading of Ras (Grosse 2000b). In the present work we provide evidence that MMP2/MMP9 activities are necessary for GTP-loading of Ras in αT3-1 cells. Gelatinase inhibitor Ro28-2653 reduced the levels of Ras-GTP raised by GnRH- or TPA challenge. These data indicate that GnRH-induced Ras activation in αT3-1 cells proceeds via a gelatinase-dependent pathway.

The gelatinase inhibitor Ro28-2653 exemplifies a new class of pyrimidine-2,4,6-trione MMP inhibitors. Batimastat is a hydroxamic acid derivative exhibiting broad-spectrum inhibitory activity and was the first metalloproteinase inhibitor to enter clinical testing (Wojtowicz-Praga 1996). As opposed to Ro28-2653, batimastat at concentrations up to 50 µM displayed only minor inhibitory effects on metalloproteinase-mediated

mitogenic signaling in gonadotropic cells. An optimized specificity of Ro28-2653 for gelatinases *in vivo* may offer one explanation for these discrepancies. Both batimastat and Ro28-2653 chelate the zinc ions in the catalytic core of metalloproteinases, but Ro28-2653 additionally forms four hydrogen bonds yielding a nearly perfect fit towards MMP2 and MMP9 (Grams 2001). Furthermore, batimastat is characterized by an exceptionally poor water solubility, thus favoring the access to cells of more soluble compounds such as Ro28-2653. Thirdly, gelatinases which belong to the most abundant MMPs in a variety of tissues, may in fact represent the vast majority of MMPs secreted by αT3-1 cells.

To address the issue of specificity of the pharmacological approach of gelatinase inhibition, we turned our attention to other members of the MAPK family known to be activated by GnRH (Naor 2000): p38MAPK and JNK are primarily known to be stimulated by environmental stress and inflammatory cytokines (Shaulian 2002). Our data using AG1478, Ro28-2653 or batimastat show that gelatinase-triggered EGFR activation is not involved in GnRH-induced p38MAPK or JNK activation in αT3-1 cells. Furthermore, the pharmacological tools used allow for selective targeting of GnRH-dependent ERK activation without unspecifically affecting other MAPK cascades.

So far, the physiological consequences of GnRH-mediated EGFR transactivation have not been investigated. Agonist occupation of the GnRH receptor in αT3-1 cells results in MAPK activation and increased mRNA and protein levels of the immediate early gene products c-jun and c-fos (Cesnjaj 1994, Mulvaney 1999) as well as in transcriptional activation of the common α subunit gene through a pathway involving PKC (Reiss 1997, Roberson 1995, Sundaresan 1996). In congruence with our data, c-fos induction has been reported to occur 1 h after agonist challenge in gonadotropic αT3-1 cells and LβT2 cells (Liu 2002, Mulvaney 1999). In the present study, we show that GnRH-mediated EGFR activation is an indispensable step in the induction of c-fos and c-jun and that MMP2 and MMP9 play a pivotal role in GnRH-dependent gene induction.

In microarray studies (Wurmbach 2001, Yuen 2002), c-jun and c-fos together with a select array of other transcription factors were among those genes found to be robustly induced by GnRH after 1 h. At present we do not know whether the induction of transcription factors other than c-jun and c-fos is also influenced by EGFR transactivation in gonadotropes. The relative contribution of different MAPK cascades engaged by the GnRH receptor and of different transcription factors to gonadotropin expression in the pituitary is discussed controversially and appears to differ

depending on the cell model under study (Harris 2002, Liu 2002, Mulvaney 1999, Naor 2000).

The results presented herein define a new role for gelatinases in the endocrine regulation of the pituitary. We show for the first time that gelatinases are activated via an inside-out signaling mechanisms by $G_{q/11}$-coupled receptors and thus we identify hitherto unknown signaling components of the EGFR transactivation cascade. Apart from their matrix-degrading abilities, MMP2 and MMP9 seem to be essential for GnRH-induced EGFR transactivation and gene induction in gonadotropes. Considering that gelatinases are also abundantly expressed in many human tumors, our results may shed new light on the mechanism by which agonists acting on other $G_{q/11}$-coupled receptors can promote tumor growth.

5.2. Pyk2 represents a cellular point of convergence by regulating neuropeptide-mediated cell proliferation or apoptosis of small cell lung cancer cells.

Neuropeptide-mediated engagement of the ERK cascade has been shown to play a crucial role for SCLC cell growth (Beekman 1998, Seufferlein 1996b). However, the mechanism underlying ERK activation via $G_{q/11}$-coupling neuropeptide hormone receptors in SCLC cells is far from being understood. We have previously shown that galanin-stimulated ERK activity solely depends on $[Ca^{2+}]_i$, thus Ca^{2+} represents the central second messenger for ERK activation in SCLC cells (Wittau 2000). In this context we have identified Pyk2, known to be abundantly expressed in neuronal tissue, as an intermediate in Ca^{2+}-mediated ERK stimulation in SCLC cells (Schlaepfer 1999).

A rise in $[Ca^{2+}]_i$ is a characteristic signaling event that can be observed in neuropeptide-stimulated H69 or H510 cells (Sethi 1991). Treatment of SCLC cells with the calcium ionophore ionomycin leads to profound tyrosine phoshporylation of Pyk2, further indicating that activation of Pyk2 can be induced by elevations of $[Ca^{2+}]_i$ alone, independent of PKC activity. Pyk2 signaling has been connected to activation of the ERK, JNK and p38MAPK pathways. One of the main functions of Pyk2 is to serve as as precisely localized adaptor platform for the assembly of signaling complexes. A $p130^{Cas}$/Crk protein complex formed at the N-terminal of two proline-rich domains specifically links Pyk2 with the activation of JNK (Blaukat 1999, Dikic 1996, Sorokin 2001, Tokiwa 1996). So far, it is unknwon how the cell manages to transmit a Pyk2-dependent signal either through the ERK or the JNK cascade by

means of a common initial calcium stimulus. However, direct association of Src tyrosine kinases with Pyk2 appears to be essential for both signaling pathways (Blaukat 1999). We observed that galanin or ionomycin efficiently induced the specific formation of a Pyk2/Src complex in H69 cells. Galanin or ionomycin also rapidly stimulated Src tyrosine kinase activity in H69 cells in a $[Ca^{2+}]_i$-dependent manner, further indicating a functional role for Pyk2/Src complex formation in SCLC cells. Nevertheless, it was recently postulated that Src tyrosine kinases are not participating in the transforming phenotype of SCLC, although direct proof was not provided (Moore 1998). Our findings do not support the latter idea, because neuropeptides like galanin were found to stimulate Src tyrosine kinases, and Src tyrosine kinase activity was required for neuropeptide-mediated GTP-loading of Ras, stimulation of ERK activity and anchorage-independent proliferation of SCLC cells. Furthermore, basal Src tyrosine kinase and ERK activities in SCLC cells were high compared to assays in COS-7 cells.

In addition we found that the regulatory p85α subunit of PI3K was tyrosine phosphorylated and coprecipitated with Pyk2 in a Ca^{2+}-dependent manner. Moreover, we found PI3K activity to be a prerequisite for galanin-induced tyrosine phosphorylation of Pyk2. These results provide evidence for a direct interaction of Pyk2 with the regulatory subunit of PI3K in H69 cells.

Tyrosine phosphorylation of p85 was reported to regulate PI3K activity by several stimuli such as bradykinin or tyrosine kinases, e.g. vascular endothelial growth factor receptor and Src-like kinases (Kavanaugh 1994, Thakker 1999, von Willebrand 1998, Xie 2000). It was demonstrated that the p85 subunit was specifically coprecipitated with activated Pyk2 in monocytic cells and blood-derived macrophages. Furthermore, Pyk2 was shown to mediate bradykinin-induced NF-κB activation via PI3K in HeLa cells. These data suggest a tight synchronous functional regulation of these two kinases (Hatch 1998, Shi 2001). This is in agreement with the fact that Pyk2 contains a putative binding site for the p85 subunit of PI3K (Chen 1994, Lev 1995). Our data support a concept with PI3K and elevations of $[Ca^{2+}]_i$ leading to tyrosine phosphorylation of Pyk2 to regulate Src activity. Src-like tyrosine kinases have been demonstrated as upstream activators of the PI3K/Akt pathway in several cell systems (Craxton 1999; Datta 1996, Dong 2000, Liu 1998). Interestingly, it was shown that the PI3K/Akt pathway is constitutively activated in SCLC cells (Moore 1998). Thus, one conceivable model could be that Pyk2 functions as a multiprotein docking molecule for PI3K and Src in SCLC cells. Once Src is bound to Pyk2, it could then

stimulate PI3K for Akt activation or/and ERK activation through the Shc/Grb2 complex (Rocic 2001).

Neuropeptide-mediated ERK activation proceeds via activated Ras in cardiac fibroblasts (Murasawa 1998). Ca^{2+}-dependent RasGEFs are able to directly activate Ras (Cullen 2002). To investigate the involvement of RasGEFs in neuropeptide-mediated ERK activation RT-PCR using primer pairs for RasGRF2 and RasGRP was carried out. We focussed on these two RasGEFs because RasGRF2 is highly expressed in lung besides neuronal tissue and RasGRP/CalDAG-GEF II displays Ras preference as compared to CalDAG-GEF I (Cullen 2002, Table 1). RT-PCR yielded RasGRF2 as being expressed in SCLC cells. Since the calmodulin inhibitor calmidazolium did not show any inhibitory effect on neuropeptide-mediated GTP-loading of Ras, the direct activation of Ras via RasGRF2 after neuropeptide stimulation in SCLC cells can be expelled. By inhibiting Src activity with PP2, we showed that GTP-loading of Ras depends on Src kinase activity. In addition, Ras activation showed $[Ca^{2+}]_i$ dependence. Our data provide first evidence that RasGEFs are not involved in neuropeptide-induced Ras activation in SCLC cells and strongly indicate that Pyk2 phosphorylation and Pyk2/Src association precede GTP-loading of Ras in SCLC cells.

Cell death after overexpression of wild type Pyk2 has been observed in fibroblastic and epithelial cell lines before, while neuroendocrine PC12 cells were resistant to Pyk2 expression (Xiong 1997). Therefore we were surprised to monitor cell death of neuroendocrine H69 cells after overexpression of Pyk2-wt by a preliminary viral as well as a transient transfection approach. Furthermore, H69 cells underwent apoptosis when transfected with Pyk2-Y402F, kinase-deficient Pyk2-PKM and Pyk2-P859A. H69 cells overexpressing Pyk2-P717A retained their viability. Pyk2 can be regarded as a multidocking adaptor for a variety of different proteins including Src kinases, the Grb2/Sos complex, paxillin, $p130^{Cas}$, Graf, PSGAP and Hic-5 (Hildebrand 1996, Matsuya 1998, Ohba 1998, Ren 2001, Salgia 1996). In H69 cells, kinase activity and autophosphorylation of Pyk2 as well as association with Src kinase are not responsible for induction of cell death as demonstrated by overexpression of Pyk2-Y402 and Pyk2-PKM. These observations strengthen our previous concept of autophosphorylation of Pyk2, activation of Src kinases and Pyk2/Src complexation upon neuropeptide stimulation as necessary prerequisites for SCLC cell survival. Previously mentioned, tyrosine 402 of Pyk2 is a putative PI3K binding site (Chen 1994, Lev 1995) and as shown above, Pyk2 forms a complex with PI3K after neuropeptide stimulation indicating that PI3K aids to cell survival of SCLC cells by an effector system not yet identified. In congruence with our data, it has been

shown that coexpression of Pyk2-wt with either active Src, Akt or PI3K rescues fibroblasts from Pyk2-induced apoptosis (Xiong 1997). As reported above, a constitutively activated PI3K/Akt pathway has been described in SCLC cells (Moore 1998), further supporting our concept.

In SCLC cells, neuropeptide hormone receptors are capable to activate G proteins of the $G_{q/11}$- and $G_{12/13}$ families (Wittau 2000). It has been shown that neuropeptide receptor antagonists lead to death of SCLC cells engaging the JNK pathway via heterotrimeric $G_{12/13}$ proteins in the way of "biased agonists" (Chan 2002). Pyk2 signaling has been revealed as a part of the Rho pathway and RhoGAPs Graf and PSGAP have been suggested as mediators (Wu 2002). Graf mediates the cross-talk between FAK and Rho family members, but a function for Graf in Pyk2 signaling has not been described so far (Hildebrand 1996). It was suggested that PSGAP may be an essential protein for regulation of cytoskeletal organization by Pyk2 via RhoGTPases (Ren 2001). Graf and PSGAP interact with Pyk2 via its second proline-rich domain (Ohba 2001, Ren 2001). Our results indicate that the $G_{12/13}$/Rho pathway does not contribute to Pyk2-induced apoptosis since Pyk2-P859A, interrupted in the second proline-rich domain, did induce apoptotic cell death in H69 cells.

The fact that cells transfected with Pyk2 mutants deficient in proline 717 remained viable, points out that proteins interacting with the first proline-rich domain of Pyk2 might be involved in pathways leading to apoptotic cell death. Galanin triggered JNK activation in SCLC cells, indicating a causal link of neuropeptide-mediated Pyk2 stimulation with JNK phosphorylation. Since an inhibitor specific for Pyk2 does not exist and SCLC cells are not amenable to high efficiency transfection, we were not able to supply a direct evidence. In support with our notion, it has been shown that Pyk2 activation leads to stimulation of JNK activity by a pathway emanating from elevation of $[Ca^{2+}]_i$ in rat liver epithelial cells (Yu 1996). In transfection experiments with dominant interferring mutants of Crk and $p130^{Cas}$, Pyk2-induced JNK activation was inhibited (Blaukat 1999). Pull-down experiments revealed that Pyk2 constitutively binds $p130^{Cas}$ via its first proline-rich domain with activated Pyk2 being capable of enhancing phosphorylation of $p130^{Cas}$ (Ohba 1998, Salgia 1996). Therefore, we conclude that overexpression of Pyk2 leads to apoptosis by means of activation of the $p130^{Cas}$/Crk/JNK pathway. One could hypothesize that the JNK pathway leading to cell death via Pyk2 is rather quiescent in SCLC cells and becomes activated in response to overexpression of Pyk2. This hypothesis is emphasized by the fact that SCLC cells display basal ERK phosphorylation being responsible for anchorage-independent growth, whereas JNK activity under unstimulated conditions is not detectable.

5.3. Conclusions, implications and perspectives

It is reasonable to assume that the basic cellular equipment in each cell is essentially identical. However, each cell type harbors e. g. cell type-specific isoforms of different proteins, some proteins are expressed ubiquitously whereas others are restricted to a specific cell type. By comparing neuropeptide/$G_{q/11}$-induced mitogenic signaling pathways in SCLC and gonadotropic αT3-1 cells, distinct pathways downstream of $G_{q/11}$ were revealed. In this context, PLCβ can be viewed as a point of bifurcation by generating PKC and $[Ca^{2+}]_i$ as two second messengers. PKC and $[Ca^{2+}]_i$ then convey signals to the ERK/MAPK cascade via different tracks.

Engagement of the ERK/MAPK signaling pathway by GnRH in gonadotropic cells proceeds via PKC as a second messenger. The temporal pattern and maximal response of ERK activity critically depends on gelatinase-triggered EGFR transactivation. Here we have shown for the first time that MT1-MMP shedding and gelatinase activation are immediate responses to occupation of GnRHR by its ligand. It is an attractive hypothesis to assume that the agonist-occupied GnRHR regulates MT1-MMP function by an intracellular mechanism employing PKC. Further studies on the regulation of MT1-MMP activity in gonadotropic cells will be enlightening in this regard.

On the contrary, galanin- or bradykinin-induced activation of the ERK/MAPK cascade solely depends on $[Ca^{2+}]_i$ as a second messenger in SCLC cells. Herein, the Ca^{2+}-dependent non-RTK Pyk2 has been characterized as the key component in neuropeptide-mediated ERK/MAPK activation. Furthermore, our data provide first evidence for Pyk2 representing a cellular point of convergence determining the fate of SCLC cells. Neuropeptide-elicited rises in $[Ca^{2+}]_i$ mediate Pyk2 phosphorylation, Pyk2/Src association and stimulation of ERK activity leading to anchorage-independent growth. Overexpression of Pyk2 in SCLC cells however results in apoptotic cell death. This conundrum might be explained by desynchronized cell signaling with Pyk2-wt overexpression resulting in apoptosis. It has been demonstrated by overexpression of GTPase-deficient $G\alpha_{16}$ that desynchronization of distinct signaling pathways emanating from a ligand-bound receptor may perturb receptor-regulated mitogenic signaling leading to the inhibition of SCLC cell growth (Heasley 1996). Another explanation would be that the duration of the Pyk2 signal after overexpression is different from the one engaged by short-term neuropeptide stimulation. This situation would lead to a different temporal pattern of Pyk2 action resulting in an altered cellular response. It has been obvious for quite some time that potent inducers of cell proliferation such as c-myc also possess proapoptotic

activities. Initially this observation was explained with oncogenes forcing cells into “unprepared cell cycles by overridding cell cycle checkpoints and inducing mitotic catastrophe”. Since then, however, it was revealed that cell proliferation and cell death are molecularly distinct mechanisms and so far the precise mechanism how oncoproteins promote apoptosis remain elusive. It is hypothesized that one of the main mechanisms by which the above mentioned balance of survival and death is achieved is by coupling cellular proliferation to apoptosis. In the case of c-myc the mitogenic and proapoptotic properties seem to be genetically inseparable (Cox 2003, Evan 1998). The clarification of the mechanism underlying Pyk2-mediated induction of apoptosis will be the subject of further investigations.

6. SUMMARY

Neuropeptide hormones are a family of structurally and functionally diverse signaling molecules. Their cognate receptors are usually classified as primarily $G_{q/11}$-coupled receptors. Activation of $G_{q/11}$ proteins results in activation of PLCβ. PLCβ catalyzes the hydrolysis of PIP_2 thereby producing the two second messengers IP_3 and DAG. IP_3 regulates rises of $[Ca^{2+}]_i$ which are realized by releasing Ca^{2+} from intracellular stores or by influx of extracellular Ca^{2+} through Ca^{2+}-channels. DAG directly activates PKC isoforms. By activating different downstream target proteins, $[Ca^{2+}]_i$ and PKC stimulate distinct signaling pathways. As most neuropeptides hormone receptors interact with overlapping portfolios of G proteins, rigorous conclusions pertaining to specific signaling pathways are often impossible. In gonadotropic cells, the GnRHR is an exclusively $G_{q/11}$-coupled receptor and GnRH challenge leads to rapid activation of the ERK/MAPK cascade with the time course being regulated by EGFR transactivation. Since GnRH-mediated ERK activation in gonadotropic αT3-1 cells is strictly reliant on PKC activity, the GnRHR in gonadotropic cells was henceforth used as a model system to study PKC-dependent ERK activation. To compare a strictly PKC-dependent ERK activation pathway with one strictly dependend on $[Ca^{2+}]_i$, we resorted to the galanin- and bradykinin receptors in SCLC cells. Since neuropeptide-mediated ERK activation in these cells is solely dependent on $[Ca^{2+}]_i$, Ca^{2+}-dependent ERK activation was studied in SCLC cells.

1. EGFR transactivation proceeds via a “triple membrane-passing signal mechanism”. Herein a variety of different metalloproteinases have been identified to cleave EGFR ligand precursors. To identify proteolytic enzymes involved in GnRH-mediated signaling events, we primarily focussed on an involvement of gelatinases since MMP2 and MMP9 are highly expressed in pituitary tumors and normal pituitary tissue. By using a specific gelatinase inhibtior, Ro28-2653, GnRH-elicited ERK phosphorylation was abrogated. To confirm the prominent role of MMP2 and MMP9 in GnRH-induced ERK activation, a more specific ribozyme-based transfection approach was applied. By transiently transfecting αT3-1 cells with ribozymes directed against MMP2 or MMP9, ERK phosphorylation after GnRH challenge was inhibited. We extended our study to another gonadotropic cells line, LβT2 cells. In these cells, GnRH-elicited ERK phosphorylation was dependent on EGFR transactivation and gelatinase activity.

2. Each of the known mammalian EGF-like gene products is synthesized as a precursor protein subject to proteolytic cleavage to release a soluble growth factor. ADAM10, ADAM12 and ADAM17/TACE have been shown to participate in RTK transactivation with various EGFR ligand precursors as substrates. To examine their contribution to proteolytic cleavage after GnRH challenge, we first chose a pharmacological approach and applied a specific ADAM17/TACE inhibitor, Ro32-7315, to αT3-1 cells. Ro32-7315 pretreatment did not have any inhibitory effect on GnRH- as well as TPA-elicited ERK phosphorylation. RT-PCR of total αT3-1 RNA using primer pairs for ADAM10 and ADAM12 revealed ADAM10 expression. Downregulation of ADAM10 by ribozyme-targeting did not have any influence on GnRH-mediated ERK phosphorylation. However, scavenging of soluble HB-EGF with neutralizing antibodies completely prevented GnRH-elicited ERK phosphorylation.

3. To characterize the role of gelatinases in GnRH-induced signaling events leading to ERK activation in gonadotropic cells, their involvement in the cross-talk between GnRHR and EGFR was studied. By inhibiting MMP2 and MMP9 with Ro28-2653, GnRH-dependent EGFR transactivation was abrogated. In order to strengthen the notion that gelatinases are specifically involved in GnRH-induced EGFR activation, the expression of MMP2 and MMP9 was targeted by transient transfection of αT3-1 cells with ribozymes. GnRH-mediated EGFR phosphorylation was substantially blocked in response to transfection with ribozymes directed against MMP2 or MMP9. Again we extended our investigation to LβT2 cells. Pretreatment of cells with the gelatinase inhibitor resulted in a decline of GnRH-induced EGFR activation.

We have shown that HB-EGF is the pivotal ligand mediating ERK activation emanating from the agonist-occupied GnRHR in αT3-1 cells. To test whether HB-EGF serves to induce EGFR phosphorylation in response to GnRH stimulation, HB-EGF was trapped with a neutralizing anti-HB-EGF antibody. Neutralization of HB-EGF inhibited GnRH-induced EGFR phosphorylation. To study the role of ADAM17/TACE or ADAM10 in EGFR transactivation, Ro32-7315 was applied or cells were transiently transfected with ribozymes targeting ADAM10, respectively. Both measures did not affect GnRH-mediated EGFR phosphorylation.

4. GnRH challenge furthered the release of active MMP2 and MMP9 and increased their gelatinolytic activities within 5 min. In this context, αT3-1 cells were stimulated with GnRH and detection of MT1-MMP in cell lysates and conditioned

culture media was performed. GnRH challenge resulted in a decline of MT1-MMP in cell lysates with increasing amounts in culture media. Ribozyme-targeted downregulation of MT1-MMP or MMP2 inhibited rapid release of MMP2 or MMP9, respectively.

5. We addressed the role of Src kinases in GnRH-mediated EGFR activation by applying the Src specific inhibitor PP2 or the EGFR specific inhibitor AG1478 to αT3-1 cells. Both inhibitors induced a decline of GnRH-mediated EGFR phosphorylation whereas the EGFR inhibitor did not change Src phosphorylation elicited by GnRH challenge. To assess the contribution of gelatinases in GnRH-induced Src activation, αT3-1 cells were pretreated with Ro28-2653 which caused the inhibtion of GnRH-induced Src phosphorylation. Transfection with ribozymes against MMP2 or MMP9 confirmed the pharmacological approach. Specific inhibition of Src kinases and EGFR activity did not yield any effect on activation of gelatinases after GnRH challenge. In LβT2 cells, GnRH-induced EGFR phosphorylation was circumvented by specific inhibition of Src kinases. Inhibition of gelatinases resulted in a decline of GnRH-induced Src activation.
GnRH- as well as short-term phorbol ester treatment resulted in GTP-loading of Ras in αT3-1 cells. GnRH-stimulated Ras activation is dependent on PKC- and Src kinase activities. Furthermore, pretreatment of cells with Ro28-2653 inhibited GnRH-mediated GTP-loading of Ras.

6. GnRH stimulation of αT3-1 cells leads to phosphorylation of JNK and p38MAPK within 15 min. Activation of JNK and p38MAPK by GnRH was unaffected by EGFR- or gelatinase inhibition.

7. EGFR transactivation in αT3-1 cells is of physiological relevance since gelatinase activation and subsequent EGFR transactivation are necessary intermediate steps required for induction of transcription factors c-jun and c-fos.

8. Ca^{2+}-dependent non-RTK Pyk2 has been shown to be an intermediate in GPCR-induced ERK activation. Since Pyk2 is abundantly expressed in neuronal tissue, we examined the expression of Pyk2 in SCLC cells and were able to amplify two specific DNA fragments. Furthermore, Pyk2 expression was verified at the protein level. Neuropeptides represent the major growth factors for proliferation and metastasis of SCLC cells. Galanin- or bradykinin stimulation induced rapid tyrosine

phosphorylation of Pyk2 in SCLC cells. This effect could be fully mimicked by treatment of the cells with the calcium ionophore ionomycin. In response to neuropeptide stimulation Pyk2 associates with Src tyrosine kinases and the regulatory p85α subunit of PI3K. Pretreatment of SCLC cells with the specific Src kinase inhibitor PP2 or the specific PI3K inhibitor LY294002 prevented neuropeptide-induced Pyk2 phosphorylation. To directly monitor Src kinase activity in SCLC cells, an *in vitro* Src kinase activity assay was performed. Galanin stimulation or elevation of $[Ca^{2+}]_i$ produced enhanced Src kinase activity in SCLC cells.

9. Neuropeptide stimulation led to GTP-loading of Ras which is reliant on $[Ca^{2+}]_i$ and Src kinase activity in SCLC cells. To test the involvement of Ca^{2+}-dependent RasGEFs in neuropeptide-induced Ras activation, their expression in SCLC cells was tested by RT-PCR. Amplification products were obtained with oligonucleotides specific for RasGRF2. To elucidate the role of RasGRF2 in neuropeptide-induced GTP-loading of Ras in SCLC, RasGRF2 was indirectly inhibited by the calmodulin inhibitor calmidazolium. Calmidazolium pretreatment did not decrease neuropeptide-mediated Ras activation.

10. Galanin- or bradykinin challenge led to stimulation of ERK activity in SCLC cells which was assessed in an *in vitro* ERK activity assay. Neuropeptide-elicited ERK activation depended on Src kinase activity.

11. The ability of cells to grow anchorage-independent is a well characterized marker for their transforming potential and can be monitored by colony formation in semisolid medium. Neuropeptides stimulated growth of SCLC cells in soft agar. Anchorage-independent growth of SCLC cells is dependent on Src kinase activity. For further characterization of the involvement of Src kinases in anchorage-independent growth of SCLC cells, cell growth was monitored in a liquid culture assay. Neuropeptide challenge elicited increased cell numbers with inhibition of Src kinase activity resulting in decreased cell numbers.

12. Overexpression of wild type Pyk2 induced apoptosis of SCLC cells as was visible in characteristic features of programmed cell death like membrane blebbing, cell swelling and apoptotic bodies. Furthermore, cell death was apparent after transfection of kinase-dead Pyk2 or Pyk2 impaired in autophosphorylation and Src family SH2-binding (Pyk2-Y402F). SCLC cells transfected with Pyk2 mutants disrupted either in the first- (Pyk2-P717A) or in the second proline-rich domain (Pyk2-

P859A) behaved differently. While Pyk2-P859A overexpression induced cell death, Pyk2-P717A overexpressed did not alter SCLC cell viability. Pyk2 has been linked to the JNK pathway by complex formation with p^{130}Cas/Crk via proline 717. Since our data indicate an involvement of the JNK pathway in Pyk2-induced cell death, SCLC cells were treated with neuropeptides and JNK phosphorylation was detected. Galanin-stimulated JNK activation started within 5 min and declined after 15 min of stimulation with basal JNK phosphorylation not apparent. In contrast, SCLC display high basal levels of ERK phosphorylation.

7. ZUSAMMENFASSUNG

Mitogene Signalwege G_q-koppelnder Rezeptoren

Neuropeptide bilden eine Gruppe von strukturell und funktionell verschiedenartigen Signalmolekülen, die an heptahelikale Rezeptoren der Zellmembran binden. Heptahelikale Rezeptoren leiten nach Interaktion mit einem heterotrimeren G-Protein die Information über Signaltransduktionskaskaden ins Zellinnere weiter. Man ist sich heute darüber im klaren, daß heptahelikale Rezeptoren und G-Proteine eine wichtige Rolle bei der Regulation von Wachstum und Differenzierung, aber auch von zellulären Transformationsprozessen spielen. Einen wichtigen mitogenen Signalweg stellt die Aktivierung der *extracellular signal-regulated kinase/mitogen-activated protein kinase* (ERK/MAPK)-Kaskade dar, die klassischerweise durch Wachstumsfaktoren wie *epidermal growth factor* (EGF) oder *platelet-derived growth factor* (PDGF) initiiert wird. ERK/MAPKs können auch über G-Protein-gekoppelte Rezeptoren aktiviert werden, deren Signale auf unterschiedlichen Ebenen der Wachstumsfaktor/Ras/Raf/MEK/ERK-Kaskade einmünden. Neuropeptidhormonrezeptoren bewirken über eine Kopplung an $G_{q/11}$-Proteine eine ausgeprägte Aktivierung der ERK/MAPK-Kaskade. $G_{q/11}$-Proteine führen durch Stimulation der Phospholipase Cβ (PLCβ) zur Erhöhung der intrazellulären Calciumkonzentration ($[Ca^{2+}]_i$) und zur Aktivierung von Proteinkinase C Isoformen (PKCs). Aufgrund der Aktivierung unterschiedlicher Effektorproteine, stimulieren PKC und Ca^{2+} voneinander abgrenzbare Signalwege.

Im Rahmen dieser Arbeit sollten die Unterschiede eines streng PKC- mit einem streng Ca^{2+}-abhängigen Neuropeptid-induzierten ERK-Aktivierungsmechanismus für das Wachstum von Tumorzelllinien untersucht und verglichen werden.

In gonadotropen αT3-1-Zellen präsentiert sich der *Gonadotropin-releasing hormone*-Rezeptor (GnRHR) als ein solitär G_q-koppelnder Rezeptor. Weiter führt die Stimulation mit GnRH zu einer raschen Aktivierung der ERK/MAPK-Kaskade, wobei die Aktivierungskinetik von der GnRH-induzierten EGF-Rezeptor (EGFR)-Transaktivierung abhängig ist. Da die ERK-Aktivierung in αT3-1-Zellen strikt PKC-abhängig ist, fungierte der GnRHR im Rahmen dieser Arbeit als ein Modellsystem, um die PKC-vermittelte ERK-Aktivierung durch $G_{q/11}$-koppelnde Rezeptoren aufzuklären. Neuropeptide stellen die wichtigsten mitogenen Stimuli für kleinzellige Lungenkarzinom (small cell lung cancer, SCLC)-Zellen dar und die Neuropeptid-induzierte ERK-Aktivierung in diesen Zellen ist strikt abhängig von der Erhöhung der

$[Ca^{2+}]_i$. Zu Beginn dieser Arbeit war der molekulare Mechanismus der Neuropeptid-induzierten ERK-Aktivierung in SCLC-Zellen unbekannt und sollte im Rahmen dieser Arbeit aufgeklärt werden.

1. Die EGFR-Transaktivierung verläuft über einen *triple membrane-passing signal* Mechanismus, der die Aktivität von Metalloproteinasen und das proteolytische Spalten von EGF-ähnlichen Liganden involviert. Im Rahmen dieser Arbeit konnten erstmalig Mitglieder der Matrixmetalloproteinasen (MMPs) als proteolytische Enzyme in G-Protein-gekoppelter-Rezeptor- (G protein-coupled receptor, GPCR)- vermittelter EGFR-Transaktivierung identifiziert werden. Mit Hilfe eines spezifischen Gelatinase-Inhibitors, Ro28-2653, konnten MMP2 und MMP9 als proteolytische Enzyme in der GnRH-vermittelten Signaltransduktion in αT3-1-Zellen identifiziert werden. Die pharmakologisch-generierten Ergebnisse wurden durch einen transienten Transfektionsansatz mit MMP2- oder MMP9-spezifischen Ribozymen untermauert. Eine 5-minütige GnRH-Behandlung setzte in αT3-1-Zellen aktive MMP2 und MMP9 ins Kulturmedium frei und führte zu einer Erhöhung ihrer Gelatine-spaltenden Aktivität. Weiter kam es nach GnRH-Stimulation zu einer Zunahme von *membrane type* (MT)1-MMP im Kulturmedium, wobei der Gehalt von MT1-MMP in Zelllysaten abnahm. Die Herunterregulation von MT1-MMP oder MMP2 durch spezifische Ribozyme verhinderte die Freisetzung von MMP2 bzw. MMP9. Mittels pharmakologischer- sowie biochemischer Daten konnte *Heparin-binding epidermal growth factor* (HB-EGF) als EGFR-Ligand im *cross-talk* zwischen GnRHR und EGFR in αT3-1-Zellen identifiziert werden.

2. Es konnte mittels pharmakologischer und biochemischer Daten aufgeklärt werden, daß die GnRH-induzierte GTP-Beladung von Ras-Proteinen und die ERK-Phosphorylierung in αT3-1-Zellen von Gelatinase-aktivierter EGFR-Transaktivierung abhängig ist. Weiter bewirkte GnRH-Stimulation eine Gelatinase-vermittelte Phosphorylierung von Src-Kinasen, die nicht EGFR-abhängig war. Aus diesen Daten kann geschlossen werden, daß Src-Kinase-Phosphorylierung in αT3-1-Zellen der EGFR-Aktivierung vorgeschaltet ist und diese reguliert.
Die Stimulation von αT3-1-Zellen mit GnRH führte zu einer Phosphorylierung von *c-jun N-terminal kinase* (JNK) und p38MAPK, jedoch waren diese Ereignisse unabhängig von Gelatinase- und EGFR-Aktivitäten.

3. Der cross-talk zwischen GnRHR und EGFR ist von physiologischer Relevanz in αT3-1-Zellen, denn es konnte gezeigt werden, daß es nach GnRH-Stimulation zu einer Induktion der Transkriptionsfaktoren c-jun und c-fos kommt, die von Gelatinase-vermittelter EGFR-Transaktivierung reguliert wird. Auch in einer weiteren gonadotropen Zelllinie, LβT2-Zellen, konnte die Beteiligung von Gelatinasen und EGFR-Transaktivierung in der GnRH-vermittelten Signalweiterleitung gezeigt werden.

4. Für die Neuropeptid-vermittelte Signaltransduktion in SCLC-Zellen konnte im Rahmen dieser Arbeit die Ca^{2+}-abhängige Nichtrezeptortyrosinkinase Pyk2 als ein bedeutendes Intermediat identifiziert werden. Sowohl in RT-PCR-Experimenten als auch in Western Blot-Analysen konnte Pyk2 als in SCLC-Zellen hoch exprimiert gezeigt werden.
Die Stimulation mit Galanin oder Bradykinin sowie die Erhöhung der $[Ca^{2+}]_i$ durch ein Calciumionophor bewirkten eine verstärkte Tyrosinphosphorylierung von immunpräzipitiertem Pyk2. Neuropeptid-Stimulation oder die Erhöhung der $[Ca^{2+}]_i$ führten zu einer gesteigerten Src-Kinase-Aktivität in *in vitro* Src-Kinase-Aktivitäts-Assays und zu einer Pyk2/Src-Kinase-Assoziation. Weiter kam es zu einer Neuropeptid-abhängigen Komplexbildung von Pyk2 mit der regulatorischen p85α-Untereinheit der Phosphatidylinositol 3-Kinase. Mit Hilfe eines GST-RBD-Fusionsproteins, bestehend aus der minimalen Ras-Bindungsdomäne (RBD) von Raf-1 und Glutathion-S-Transferase (GST), konnte in einem Ras-Aktivierungs-Assay erstmalig in SCLC-Zellen die Neuropeptid-vermittelte GTP-Beladung von Ras-Proteinen gezeigt werden, die sich $[Ca^{2+}]_i$- und Src-Kinase-abhängig präsentierte. Anhand von RT-PCR-Experimenten und pharmakologischen Daten konnte der Einfluß von Ras-Austauschfaktoren ausgeschlossen werden, so dass die Daten auf eine direkte Pyk2/Src-Kinase-vermittelte Ras-Aktivierung schließen lassen.
Die Neuropeptid-stimulierte Erhöhung der ERK-Aktivität stieg in Abhängigkeit von Src-Kinase-Aktivität, was in *in vitro* ERK-Kinase-Aktivitäts-Assays gezeigt werden konnte. In *Soft-Agar*- und *Liquid-Growth*-Assays wurde die biologische Bedeutung des Pyk2/Src-Kinase-Signalweges für das Wachstum von SCLC-Zellen unterstrichen. Die Inhibition der Src-Kinase-Aktivität führte zu einer drastisch verminderten Koloniebildung nach Galanin- oder Bradykinin-Stimulation im semisoliden Medium und einem verringerten Wachstum im Flüssigmedium.

5. Überraschenderweise induzierte die *Überexpression von Pyk2* in SCLC-Zellen Apoptose, die an Membranblasenbildung, Zellschwellung oder apoptotischen Zellkörperchen sichtbar wurde. Weiter kam es nach Überexpression einer Kinase-defizienten- (Pyk2-PKM) sowie einer Autophosphorylierungs-beeinträchtigten (Pyk2-Y402F) Pyk2-Mutante zu programmiertem Zelltod. Zwei weitere Pyk2-Mutanten, die in der ersten- (Pyk2-P717A) oder zweiten (Pyk2-P859A) Prolin-reichen Region Mutationen aufweisen, zeigten bezüglich der Induktion von Apoptose ein unterschiedliches Verhalten. Während es nach Überexpression von Pyk2-P859A zu einem Absterben von SCLC-Zellen kam, verursachte die Transfektion mit Pyk2-P717A keine Apoptose. Es ist gezeigt worden, daß Pyk2 via Prolin 717 einen Komplex mit dem Adaptorprotein p^{130}Cas ausbildet und so in die JNK-Kaskade einmündet. Nach Neuropeptid-Stimulation kam es in SCLC-Zellen zu einer Erhöhung der Phosphorylierung von JNK, wobei eine basale Phosphorylierung nicht apparent war. Im Gegensatz dazu, zeigte die ERK-Phosphorylierung in diesen Zellen eine hohe basale Phosphorylierung.

Zusammenfassend läßt sich sagen, daß in Abhängigkeit vom untersuchten Zellmodell das Neuropeptid/$G_{q/11}$-initiierte Signal auf unterschiedlichen Ebenen in die Ras/Raf/MEK/ERK-Kaskade einmündet. In gonadotropen αT3-1-Zellen kommt es auf Ebene des EGFR zu einer Einmündung des GnRHR/$G_{q/11}$-induzierten Signalweges in den ERK/MAPK-Signalweg. GnRH-Stimulation bewirkt in diesen Zellen eine erhöhte Gelatinase-Aktivität, was über die proteolytische Spaltung von HB-EGF zur Aktivierung des EGFR führt. Die EGFR-Transaktivierung besitzt in diesem Zellmodell eine physiologische Relevanz, da es in Abhängigkeit von Gelatinase-induzierter EGFR-Aktivierung zu einer Induktion von Transkriptionsfaktoren kommt. In SCLC-Zellen kommt es über die Neuropeptid-vermittelte Erhöhung der $[Ca^{2+}]_i$ zur Phosphorylierung der Nichtrezeptortyrosinkinase Pyk2. Pyk2 stellt in diesen Zellen ein zentrales Regulationsprotein dar. Einerseits kommt es über Assoziation mit Src-Kinasen zu einer Aktivierung des Ras/Raf/MEK/ERK-Signalweges, der für das Wachstum dieser Tumorentität von entscheidender Bedeutung ist. Andererseits kommt es nach Überexpression von Pyk2 zu einer erhöhten Apoptoserate. Diese Ergebnisse identifizieren Pyk2 als neue, interessante therapeutische Zielstruktur für die Behandlung des kleinzelligen Lungenkarzinoms.

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10. CURRICULUM VITAE

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Education

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Academic education and scientific work

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11. PUBLICATIONS

11.1. Original publications generated within the scope of the thesis

Grosse R, Roelle S, Herrlich A, Hohn J, Gudermann T. 2000. Epidermal growth factor receptor tyrosine kinase mediates Ras activation by gonadotropin-releasing hormone. J Biol Chem. 275: 12251-12260

Roelle S, Grosse R, Aigner A, Krell HW, Czubayko F, Gudermann T. 2003. Matrix metalloproteinases 2 and 9 mediate epidermal growth factor receptor transactivation by gonadotropin-releasing hormone. J Biol Chem. 278: 47307-47318

Roelle S, Grosse R, Chubanov V, Stöppler H and Gudermann T. 2004. Pyk2 represents a molecular switch for proliferation versus apoptosis of small cell lung cancer cells; submitted

11.2. Contributions to congresses

Roelle S, Grosse R, Wittau N, Hofmann T, Schultz G, Gudermann T.
Calcium-mediated activation of Pyk2 and Src tyrosine kinases promotes growth of small cell lung cancer cells through G-protein-coupled receptors
Wissenschaftswoche 1999, Universitätsklinikum Benjamin Franklin der Freien Universität Berlin, Jahrbuch 1999

Grosse R, Roelle S, Hofmann T, Wittau N, Schultz G, Gudermann T.
Rolle von Pyk2 und Src-Tyrosinkinasen für das neuropeptid-gesteuerte Wachstum von kleinzelligen Bronchialkarzinomzellen
41. Frühjahrstagung der Deutschen Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie in Mainz

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Galanin-mediated activation of Pyk2 and Src tyrosine kinases promotes growth of small cell lung cancer cells
35. Jahrestagung der Deutschen Diabetes Gesellschaft und 44. Symposium der Deutschen Gesellschaft für Endokrinologie

Roelle S, Grosse R, Hofmann T, Schultz G, Gudermann T.

Galanin-mediated activation of Pyk2 and src tyrosine kinases promotes growth of small cell lung cancer cells
Fall meeting of the German Society for Experimental and Clinical Pharmacology and Toxicology and Sixth International Dahlem Symposium (SFB 366)
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Roelle S, Grosse R, Höhn J, Schultz G, Gudermann T.
Epidermal Growth Factor receptor Transactivation by Gonadotropin-releasing Hormone receptor depends on Gelatinase Activity in αT3-1 cells
43. Frühjahrstagung der Deutschen Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie in Mainz, 2002

Chubanov V, Roelle S, Stöppler H and Gudermann T.
Overexpression of proline-rich tyrosine kinase PYK2 induces apoptosis in small cell lung cancer cells
43. Frühjahrstagung der Deutschen Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie in Mainz, 2002

Roelle S, Grosse R, Krell H-W and Gudermann T.
Gelatinase activity is required for Epidermal Growth Factor Receptor Transactivation by Gonadotropin-releasing Hormone in αT3-1 cells
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Roelle S, Grosse R, Aigner A, Krell H-W, Czubayko F and Gudermann T.
Matrix metalloproteinases 2 and 9 mediate epidermal growth factor receptor transactivation by gonadotropin-releasing hormone
45. Frühjahrstagung der Deutschen Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie in Mainz, 2004

Büch T, Roelle S, Kaske S, Hess C, Bierbermann H and Gudermann T.
Activation of extracellular signal-regulated protein kinase 1/2 (ERK1/2) by thyrotropin via epidermal growth factor receptor
45. Frühjahrstagung der Deutschen Gesellschaft für experimentelle und klinische Pharmakologie und Toxikologie in Mainz, 2004